Nucleotide enhancement of diets, fish reproduction and egg quality

Thesis

Nucleotide enhancement of diets, fish reproduction and egg quality

José Luis González Vecino

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Abstract

Fish reproduction, and egg and larval quality are affected by broodstock nutrition. Nucleotides, the building blocks of DNA and RNA, are now considered as semiessential nutrients during periods of food deficiency, stress, rapid growth and immunological stress. Since oogenesis is a process of intensive cell division with high nucleic acid formation and a concomitant high requirement for nucleotides, broodstock diets were enhanced with nucleotides. Their effects on the reproductive performance of Atlantic halibut (*Hippoglossus hippoglossus* L.) and haddock *Melanogrammus aeglefinus* L.), two cold water species with different life histories, were compared with control diets.

Broodstock condition at the end of the spawning seasons did not differ between dietary treatments. However, dietary nucleotides improved total relative fecundity and batch relative fecundity of halibut and haddock respectively. Egg quality was also improved in both species. Fertilisation rate of haddock eggs and hatching rate in halibut and haddock eggs were significantly higher with the nucleotide diets. Nucleotide supplementation resulted in improved larval quality in both species. Survival of halibut larvae at the end of the yolk-sac stage was higher in the nucleotide group. Haddock larval survival five days after hatch was the same in both diets but improved at ten days post hatch as a result of significantly better developed gut and first feeding success in the nucleotide group. Histological examination of fish ovaries at the end of the experiment revealed no differences in the atresia levels.

Nucleotide composition of egg and larval samples did not differ between diets, suggesting that dietary nucleotides were utilised by the broodfish to cover their nutritional requirements during oogenesis and reproduction. The enhanced reproductive performance in the nucleotide group is discussed in relation to a higher lipid mobilisation during oogenesis and quality of the yolk being consequently improved. Energy charge is confirmed as an egg quality indicator.

To my family

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Glossary

A or Ado:	Adenosine
Ade:	Adenine
ADP:	Adenine diphosphate
AMP:	Adenine monophosphate
ANCOVA:	Analysis of covariance
ANOVA:	Analysis of variance
Apo:	Apolipoprotein
ARA:	Arachidonic acid (20:4n-6)
ATP:	Adenine triphosphate
BM:	Blastomere morphology
BN:	Batch number
C or Cyd:	Cytidine
cAMP:	cyclic AMP
cGMP:	cyclic GMP
CoA:	Coenzyme A
Cyt:	Cytosine
DA:	Dopamine
DHA:	Docosahexaenoic acid (22:6n-3)
DNA:	Deoxyribonucleic acid
EC:	Energy charge
EM:	Egg wet mass
EPA:	Eicosapentanoic acid (20:5n-3)
EV:	Egg volume
FAD^+:	Flavinadenine dinucleotide
FMN^+:	Flavine mononucleotide
FR:	Fertilisation rate
G or Guo:	Guanosine
GLM:	General linear model
GMP:	Guanosine monophosphate
GnRH:	Gonadotropin-releasing hormone
GtH:	Gonadotropin
Gua:	Guanine
HDL:	High density lipoprotein
HR:	Hatching rate
HUFA:	Highly unsaturated fatty acid
Hyp:	Hypoxanthine
I or Ino:	Inosine
Ig:	Immunoglobulin
IPN:	Infectious pancreatic necrosis
IPNV:	Infectious pancreatic necrosis virus
LCAT:	Lecithin-cholesterol-acyl-transferase
LDL:	Low density lipoprotein
NAD^+:	Nicotinamide adenine dinucleotide
$NADP^+$:	Nicotinamide adenine dinucleotide phosphate
NBT:	Nitroblue tetrazolium
NDP:	Nucleotide diphosphate
NK:	Natural killer cell
NMP:	Nucleotide monophosphate
NS:	Nucleoside

NT:	Nucleotide
NTP:	Nucleotide triphosphate
PGI:	Prostacyclin
PUFA:	Polyunsaturated fatty acids
Pur:	Purine
Pyr:	Pyrimidine
R or Puo:	Purine nucleoside
RNA:	Ribonucleic acid
SGA:	Small for gestational age
T or Thd:	Thymidine
Thy:	Thymine
TMP:	Thymine monophosphate
TPN:	Total parenteral nutrition
TXA:	Tromboxane
U or Urd:	Uridine
UDP:	Uridine diphosphate
Ura:	Uracil
VLDL:	Very low density lipoprotein
VTG:	Vitellogenin
WC:	Water content
Y or Pyd:	Pyrimidine nucleoside

Chapter 1: General introduction

Worldwide overfishing has raised concerns that extraordinary collapses of fish stocks may significantly increase the extinction probability of marine species both targeted (IUCN, 1996) (for example, Atlantic cod, *Gadus morhua*) and incidentally harvested (Casey & Myers, 1998) (for example, barndoor skate, *Raja laevis*). This global decline of ocean fisheries stocks has provided impetus for the development of aquaculture. The contribution of aquaculture to global supplies of fish, crustaceans and molluscs continues to grow, increasing from 3.9 percent of total production by weight in 1970 to 27.3 percent in 2000 according to FAO statistics. Aquaculture is growing more rapidly than all other animal food producing sectors(FAO, 2002a).

1.1 Broodstock management

Broodstock are one of the most important but often overlooked production stages in aquaculture. Currently, difficulties in the supply of eggs and larval fry are amongst the most important constraints on current and future aquaculture development (Bromage, 1995). From the 153 major finfish species cultivated (FAO, 2002b), there is little and in many cases no information on broodstock management. Ideally broodfish should be maintained under controlled conditions matching as far as possible or improving those which the fish would have been exposed to in the wild. However, in practice, it is very difficult to manage all the rearing conditions. Broodstock should have optimal growth to maturity, be capable of undergoing maturation (physiologically and/or behaviourally) at the optimal time (developmentally and seasonally), have a high likelihood of surviving through spawning and produce gametes in optimal numbers, of optimal sizes (in the case of eggs), and of optimal quality (Bromage, 1995). The genetic make-up of the fish provides the blueprint on which these performance characteristics are based. In fact, the use of selective breeding programmes are now carefully considered by egg and larval providers (see Brooks *et al.* (1997) regarding genetic influences on

egg quality). However, specific conditions such as feeding regime and diet (Watanabe, 1985), environmental conditions (Bromage, 1995), exposure to pathogens and handling stress (Schreck *et al.*, 2001) are all known to affect fish reproduction. Research progress in these areas is slow because of the relatively short history of work on reproduction in many species, particularly marine fish, and because of the multiplicity of interrelated factors which are thought to act on egg and larval quality in fish. Furthermore, the necessity of suitable in-/outdoor culture facilities for maintaining large groups of adult fish and consequently the higher costs of conducting broodstock feeding trials, compared to larval ones, also constrain this type of study. Improvements in the understanding of the appropriate culture conditions, management procedures and nutritional requirements of the broodfish are essential to control reproductive development and produce viable eggs and larvae.

1.2 Biology of Atlantic halibut and haddock

Two species with different life-histories were selected in order to study the effects of nucleotide supplementation on broodstock nutrition. Atlantic halibut (*Hippoglossus hippoglossus*) and haddock (*Melanogrammus aeglefinus*) are two marine fish with potential for aquaculture but with differing biological development. Halibut is a flatfish with slow growth rates, a long larval yolk sac stage and experiences drastic changes during its metamorphosis to become juveniles, whereas haddock is a gadoid with faster growth rates, a much shorter yolk-sac stage and does not suffer metamorphic changes as drastic as those of flatfish. The general characteristics of the biology of the two species used in this project - Atlantic halibut and haddock- are described in this section.

1.2.1 Atlantic Halibut

Atlantic halibut, *Hippoglossus hippoglossus*, L. (1758) is the largest of the Pleuronectidae family, which includes other flatfish such as sole (*Solea solea*), plaice (*Pleuronectes platessa*),

Pacific halibut (*H. stenolepis*) and Japanese flounder (*Paralichthys olivaceus*) among many others. Atlantic halibut is found in the North Atlantic from the Barents Sea and Greenland as far south as the Bay of Biscay and New York on the eastern and western sides respectively. It is one of the most highly prized edible fish, due to its high flesh quality. It is territorial, grows very slowly and matures late. Furthermore, it is classified as an endangered species facing a very high risk of extinction in the wild, with declining populations (IUCN 2002).

A broad description of the biology of this species was reviewed by Haug (1990). Immature and mature halibut occupy different habitats in the wild. Coastal waters (20 – 60 m depth) may serve as nursery areas which juvenile halibut inhabit for the first 4 – 6 years. Intense migration to both shallow and deep waters seems to occur when leaving the nursery areas. Halibut congregate for spawning in winter on well-defined deepwater spawning grounds where, almost exclusively, sexually mature fish occur. Male Atlantic halibut reach sexual maturity at a younger age than females, so the latter are usually larger in size due to this late maturation. Björnsson (1995) proposed that these different growth patterns between males and females are due to natural selection. Small males can produce sufficient amounts of milt to ensure fertilisation whilst female fecundity increases with increased body weight. In practice it has been found that maturity is not reached before 6-7 years of age. It is widely believed that first maturation is mainly related to the weight of the fish but experience in hatcheries has shown that the age is a significant factor as well (Peter Smith SAMS*Ardtoe*, pers. comm.). Atlantic halibut spawn during one season that extends approximately from March until June with a peak in April-May. It is a batch spawner; females do not release all the eggs in once, but in sequential batches every 3-4 days. The number of eggs per batch and per spawning season are closely related to the size of the fish and the egg yield might equal 40% of the body mass under favourable conditions (Mangor-Jensen *et al.*, 1998a).

With the expansion of aquaculture in Europe over the last 20 years, there has been a growing interest in the farming of new species such as halibut. Norway was the first country that attempted its cultivation. Atlantic halibut research in the UK started in 1983 at the Seafish

Aquaculture (now SAMS*Ardtoe*) and presently commercial cultivation is conducted at three operational hatcheries (including SAMS*Ardtoe*) and four ongrowing sites in the U.K.. However, although halibut farming has been investigated in Norway and the UK for many years, two main bottlenecks remain:

- Egg-quality has not been consistent over time, with very poor batches on some occasions. This issue is currently being addressed by manipulating broodstock diets (e. g. Alorend *et al.*, 2003).
- Larval mortality is very high during the transition from endogenous to exogenous feeding. Procedures to reduce it have been recently investigated through the LINK-Aquaculture – FIN 22 project (Batty *et al.*, 2003).

1.2.2 Haddock

Haddock, *Melanogrammus aeglefinus*, L. (1758) is a member of the Gadidae family, which also includes other commercially important fish such as cod (*G. morhua*), pollack (*Pollachius pollachius*) and blue whiting (*Micromesistius poutassou*). Haddock are bentho-pelagic fish and are found off the coast of Northern Europe, the British Isles and Iceland, and in waters along eastern North America. Haddock are especially prevalent in areas off the coast of Newfoundland and Nova Scotia, as well as areas of the Gulf of Maine and George Bank. Landings throughout the Atlantic have declined substantially in the last 40 years and as a result this species is classified as vulnerable and facing a high risk of extinction in the wild in the medium-term future (IUCN, 2002).

Haddock mature at about 3 – 4 years of age and the proportion of mature individuals increases gradually with age. Males mature at a slightly younger age and at a smaller size than the females. Haddock migrate to deep spawning grounds and spawning occurs mainly during March - May. No field observations have been made of the spawning behaviour, but in captive haddock, the males perform a courtship ritual involving the erection of the unpaired fins, production of sounds (Hawkins & Amorim, 2000) and in some cases also changes in

colouration. Males swim belly-up under the female and although fertilisation is external its efficiency is increased by a pattern of mating behaviour that terminates in a spawning embrace, bringing both gonopores close (Hislop, 1984; Trippel *et al.*, 1998). As with halibut, the haddock is a batch spawner and the females produce batches of eggs. Periodicity with which these egg batches are spawned (inter-batch interval) was reported to be 2 – 4 days (Hislop *et al.*, 1978), although recent studies from Trippel *et al.* (1998) demonstrated that small batches are released at short intervals of 2 – 4 days, with longer intervals between large batches. The batch size and number of batches released along the season – around 8-10 –depends on fish size.

Haddock has been targeted as a promising new species for aquaculture and since the late 1990's its research has been conducted in Norway, Canada, USA and the UK. However the research on haddock farming and its nutritional requirements is still very limited. Seafish Aquaculture pioneered research on this species in 1999 and currently leads it in the UK. As with halibut, the husbandry of haddock during this project was also carried out at this laboratory.

1.3 Fish reproduction

Securing the availability of viable gametes is essential in aquaculture and there are mainly two routes to achieve it: the collection of naturally spawned eggs or the stripping of gametes from broodfish. In either case the origin of the gametes could be directly from the wild; from wild fish acclimated as broodstock or from hatchery-reared mature fish. As mentioned above, fish reproduction is sensitive to nutrition, environmental and social conditions and stress. Therefore, understanding of the basic reproductive processes occurring in sexually mature fish is required.

Oocyte development

Germinal tissue in the developing gonad differentiates into spermatogonia or oogonia. Oogonia develop into oocytes, which in all teleosts appears to undergo the same basic pattern of growth, regardless of their reproductive strategy. Oocyte development can be broadly divided into six

phases according to their state of growth: oogenesis, primary oocyte growth, the cortical alveolus stage, vitellogenesis, oocyte maturation and ovulation (Nagahama, 1983). The onset of gonadal maturation is determined by environmental factors and regulated by the endocrine system via the hypothalamus-pituitary-gonadal axis (Figure 1. 1). The pituitary gland is stimulated or inhibited by hypothalamic compounds such as gonadotropin-releasing hormone (GnRH) and dopamine (DA) respectively. As a result of this GnRH induces the formation of two gonadotropins (GtH), GtH $_{I}$ and GtH $_{II}$, which are secreted into the plasma and act at different stages (Swanson, 1991). GtH $_{I}$ affects the ovarian follicle cells and stimulates the synthesis of 17β- oestradiol (Suzuki *et al.*, 1988), which in turn leads to the production in the liver of vitellogenin (VTG) (Ng & Idler, 1983) and egg shell proteins (Oppen-Berntsen *et al.*, 1994). These are sequestered by the ovary and then incorporated into the oocytes. This is when most energy reserves are taken up and stored for future use by the developing embryo. As a consequence of the uptake of compounds by the oocyte, vitellogenesis is therefore the principal event responsible for the enormous growth of fish oocytes. At the end of vitellogenesis, post-vitellogenic follicles undergo final oocyte maturation. GtH $_{II}$ affects this stage, acting on the follicle cells to stimulate the production of progesterones, which control the termination of oocyte growth and ovulation of the egg (Suzuki *et al.* 1988). In marine species with pelagic eggs, there is a final increase in size as oocytes hydrate just prior to ovulation; this may be accompanied by the final formation of one or more lipid droplets (Wallace & Selman, 1981). A review on oocyte development in teleosts was carried out by Tyler & Sumpter (1996).

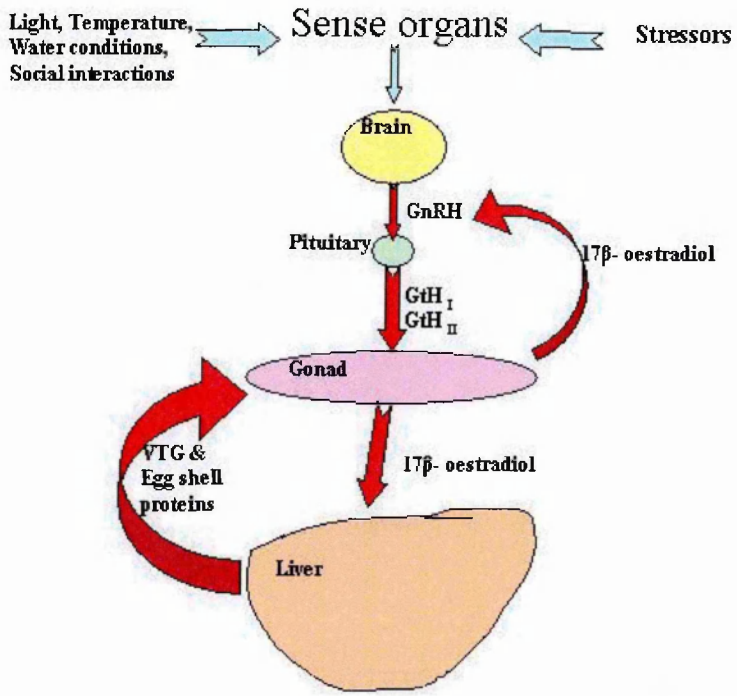


Figure 1. 1 Schematic representation of the hypothalamus-pituitary-gonad-liver (HPGL) axis. The HPGL is regulated through the negative feedback mechanism by 17β- oestradiol. GnRH: gonadotropin releasing hormone; GtH: gonadotropin I & II.

There are three commonly recognised modes of oocyte development within the basic developmental pattern described above (Wallace & Selman, 1981):

- **Synchronous:** This is characterised by a single clutch of oocytes, all developing and ovulating at the same time; further replenishment from earlier stages does not take place. This is found in semelparous species (that spawn only once and then die) such as catadromous eels or anadromous salmon species (*Salmo* and *Oncorhynchus* spp).

- **Group – synchronous:** This is chracteristic of iteroparous species (that spawn more than once in their lifetime). Here the ovary contains at least two populations of oocytes; one containing larger developing oocytes (defined as a "clutch") destined for maturation and ovulation during the current spawning season, and a second group of

previtellogenic oocytes for future seasons. This is typically found in Atlantic cod (*G. morhua*), haddock (*M. aeglefinus*) and Atlantic halibut (*H. hippoglossus*) among others.

- **Asynchronous:** Oocytes of all stages of development are present without dominant populations. The ovary appears to be a random mixture of oocytes at different stages. It is only possible to separate stocks of oocytes when hydration occurs. European hake (*Merluccius merluccius*) and Atlantic mackerel (*Scomber scombrus*) and small pelagic temperate species in general follow this pattern.

Spermatogonia development

Spermatogonia divide mitotically into primary spermatocytes which then undergo meiotic divisions to form secondary spermatocytes and the division of these will produce spermatids. This transition from spermatogonia into spermatid is called spermatogenesis, with the further development of spermatids into spermatozoa referred to as spermiogenesis (Nagahama, 1983). Spermatozoa are released into the sperm ducts (spermiation) and the gamete development culminates with the hydration of the seminal fluid-spermatozoa suspension (milt) (reviewed by Pankhurst (1994)). The patterns of gamete synchrony in males mirror those previously described for females above: synchronous, group synchronous and asynchronous.

Oogenesis and spermatogenesis are therefore characterised by intensive cell divisions. Both mitosis and meiosis involve a high level of DNA replication and RNA synthesis. In fish, although there is little information available, a period of intense RNA synthesis occurs during the initial stages of primary growth –previtellogenic growth- (Wallace & Selman, 1990). During primary growth there is an increase of at least one order of magnitude in oocyte volume in fish (Nagahama, 1983; Wallace & Selman, 1990). This growth is accompanied by the generation of increasing numbers of cytoplasmic organelles, including mitochondria, multivesicular bodies, the endoplasmic reticulum and Golgi complex (Bromage & Cumaranatunga, 1988). This growth activity is supported by very intense ribosomal and RNA activity, with numerous nucleoli present in the nucleus (de Vlaming, 1983). RNA activity again plays a major role in mediating

the regulation of final maturation, by transcribing for different enzymes acting on steroids in follicular cells (Nagahama, 1994). Therefore, a limited availability of nucleotides may compromise this complex "machinery". In the present study it is hypothesised that increasing the availability of nucleotides through the broodstock diet before oogenesis starts may be beneficial for the reproductive performance of the fish. More detailed information on nucleotide structure and function is described in the following section.

1.4 Nucleotides

1.4.1 Nomenclature and definition:

All nucleotides (NT) consist of three components (Figure 1. 2):

1) Nitrogenous base which can be either a purine or pyrimidine
2) Pentose sugar: A five-carbon sugar is linked to the base. The pentose is either ribose or deoxyribose and subsequently forms riboNT or deoxyriboNT.
3) Phosphate ester: Phosphate groups are attached to the sugar as esters, with the most common site of the esterification in natural compounds being via the hydroxyl group at C5'. Typically, one, two or three phosphates are joined, producing mono-, di- and triphosphates respectively.

Nucleosides (NS) have very similar structures to NTs, consisting only of the base bonded to the sugar (Figure 1. 2). Thus, a NT is usually referred as a phosphate ester of a NS.

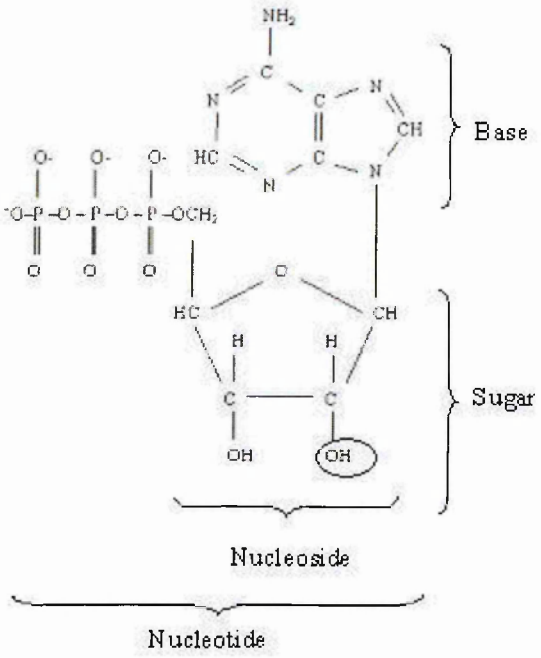


Figure 1. 2 Chemical structure of adenosine triphosphate (ATP) nucleotide.

Terminology and nomenclature for NT, NS and their related compounds are complex so standard nomenclature has been suggested to aid understanding (Table 1. 1). Nucleotides are referred to usually in abbreviated form. For example, molecules with one, two and three phosphates attached to adenosine are AMP (adenosine monophosphate), ADP (adenosine diphosphate) and ATP (adenosine triphosphate); the respective deoxy variants are dAMP, dADP and dATP. Nucleotides without specified bases are NMP, NDP and NTP. Nomenclature in general use is listed in Table 1. 1

Table 1. 1 Nomenclature of bases, nucleosides and nucleotides. Designation of the deoxyribose forms of the nucleotides is done by prefixing with deoxy (or d for abbreviated forms). Abbreviated notations for multiple phosphorylated nucleotides use D for diphosphate and T for triphosphate

Base	Ribonucleoside	Ribonucleotide	Deoxy-ribonucleotide
Purines (Pur)	Purine nucleoside (R or Puo)		
Adenine (Ade)	Adenosine (A or Ado)	AMP	dAMP
Guanine (Gua)	Guanosine (G or Guo)	GMP	dGMP
Hypoxanthine (Hyp)	Inosine (I or Ino)	IMP	
Pyrimidines (Pyr)	Pyrimidine nucleoside (Y or Pyd)		
Cytosine (Cyt)	Cytidine (C or Cyd)	CMP	dCMP
Thymine (Thy)	Thymidine (T or Thd)	TMP	dTMP
Uracil (Ura)	Uridine (U or Urd)	UMP	

In addition to their monomeric state, NTs exist in polymeric forms called nucleic acids. The different riboNTs and deoxyriboNTs link by 5'-3' phosphodiester bonds and form ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) respectively.

Apart from being the major components of the nucleic acids, NTs are organic compounds that play a key role in most intracellular biochemical processes (Table 1. 2). Nucleotides can be salvaged from degraded nucleic acids and nucleotides and also synthesised *de novo* by the body utilising amino acid precursors. Therefore, they are not considered essential nutrients; in fact, no particular disease has been related to a NT deficiency. However, evidence suggests that when the endogenous supply is insufficient, such as periods of rapid growth, certain disease states, stress and limited nutrient intake, a lack of dietary NTs may impair important functions. This suggests a key nutritional role. For this reason NTs are now considered as semi- or conditionally essential nutrients (Uauy, 1989; VanBuren & Rudolph, 1997; Sánchez-Pozo & Gil, 2002).

1.4.2 Nucleotide functions:

All living cells contain a variety of NTs and they participate in many metabolic functions (Cosgrove, 1998):

1) Energy metabolism: NTPs act as energy-rich precursors of mononucleotide units by losing their terminal phosphate groups. ATP is the universal intracellular currency of energy in biological systems. It acts as a phosphate carrier in many important enzymatic reactions involved in the transfer of energy. Furthermore, energy charge (EC), a ratio expressing the proportions of the different adenine nucleotides, controls energy metabolism (Atkinson & Walton, 1967, Chapter 5).

2) Nucleic acid precursors: As mentioned above, NTs are the monomeric units of DNA and RNA, which are crucial for the storage, transfer and expression of genetic information.

3) Physiological mediators: NTs and derivatives serve as mediators of many metabolic processes. For example, cyclic AMP (cAMP) and cyclic GMP (cGMP) are "second messengers" in the action of many hormones. ADP is critical for normal platelet aggregation. GTP is involved in signal transduction, RNA structure and microtubule formation.

4) Coenzyme components: NT's are structural components of coenzymes that are crucial to many metabolic pathways. For example: Nicotinamide adenine dinucleotide (NAD^+), flavinadenine dinucleotide (FAD^+) and coenzyme A (CoA). The main reactions of the nucleotide coenzymes are summarised in Table 1. 2.

5) Activated intermediates: nucleotides serve as carriers of activated intermediates for many reactions. For example, UDP-glucose in the process of glycogenesis, and UDP-galactose in the synthesis of lactose.

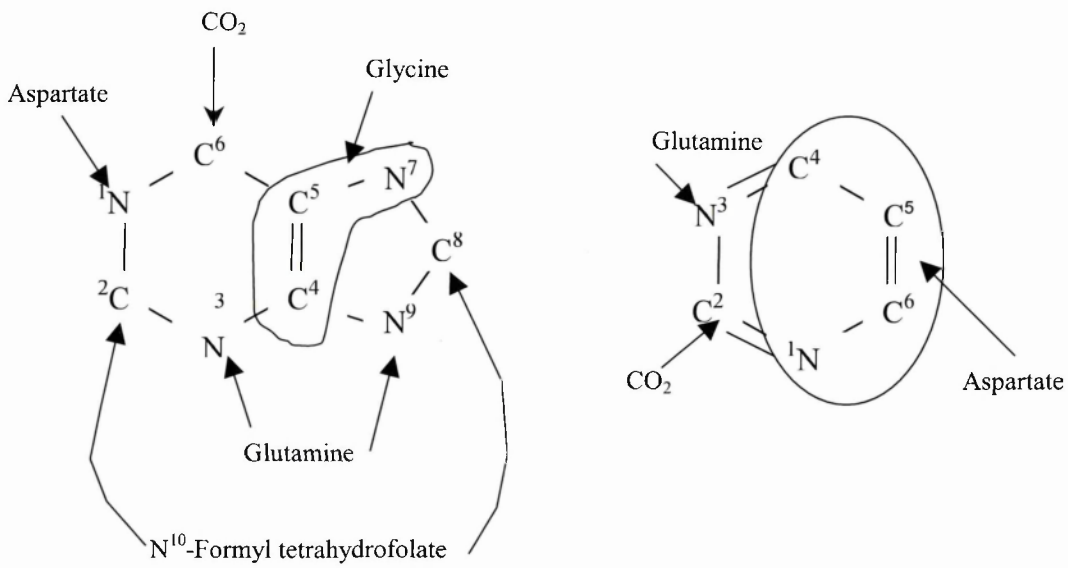
6) Allosteric effectors: intracellular concentrations of nucleotides regulate the steps of many metabolic pathways (e.g. pyrimidine biosynthesis).

Table 1. 2 Biochemical functions of nucleotide-related compounds. (Hutchinson, 1964)

Compound	*Biochemical function*
Nucleoside di- and triphosphates	Phosphorylation reactions Coenzyme synthesis Nucleic acid synthesis
Nicotinamide adenine dinucleotide (NAD^+) Nicotinamide adenine dinucleotide phosphate ($NADP^+$) Flavine mononucleotide (FMN^+) Flavin adenine dinucleotide (FAD^+)	Oxidation-reduction
Coenzyme A (CoA)	Acyl transfer Fatty acid synthesis Glyceride synthesis
Nucleoside diphosphate sugars	Polysaccharide synthesis Phospholipid synthesis Teichoic acid synthesis

1.4.3 Nucleotide metabolism:

Nucleotides and nucleic acids are constantly being formed and degraded. There are two major pathways for the formation of nucleotides: synthesised *de novo* from amino acid precursors or salvaged from degraded nucleic acids and nucleotides. Almost all the atoms in purine and pyrimidine bases are derived directly or indirectly from amino acids; the biosynthetic origin of the various atoms in both bases can be seen in Figure 1. 3.



a) Purine base b) Pyrimidine base

Figure 1. 3 Biosynthetic origin of the atoms in purine (a) and pyrimidine (b) bases.

De novo synthesis of NTs is a metabolically costly process and requires a great deal of energy in the form of ATP. Salvage pathways reuse bases and NSs to convert them into NTs. Bases, NSs and NTs come from nucleic acid degradation (intracellular or as result of cell death), digestion of nucleic acid ingested in the diet or even dietary NSs and NTs themselves. The use of dietary nucleotides is therefore an alternative mechanism to maintain the NT pool thus conserving energy. Tissues with a rapid cell turnover (such as the intestine, bone marrow and immune system) require exogenous nucleotides for synthesis of nucleic acid (Navarro *et al.*, 1996). Nucleotides become essential when the *de novo* synthesis is not able to fulfil the body needs during periods of food deficiency, rapid growth (intensive cell division) and immunological

stress (Uauy, 1989; Carver, 1994; 1999).They are therefore considered semi- or conditionally essential nutrients (Uauy, 1989; VanBuren & Rudolph, 1997; Sánchez-Pozo & Gil, 2002).

1.4.4 Research on nucleotides

Most of the research on nucleotides has studied the effects of these compounds on the gastrointestinal system, lipid metabolism, liver and immune system. The main findings in each of these areas are described in this section.

1.4.4.1 Gastrointestinal effects

The intestine and liver incorporate proportionally greater amounts of dietary NT than any other organ. Therefore a great amount of research has focussed on the effects of dietary NT supply on intestinal and hepatic morphology and function.

Intestinal growth and differentiation

Different studies have confirmed that dietary nucleotides play an important role in the development of the gastrointestinal tract. Uauy *et al.* (1990) discovered that dietary nucleosides increased mucosal protein and DNA, as well as villus height in the proximal intestinal segment of rats. The muscle of the gut was thicker and disaccharidase activities were also higher in the NT group; similar results were also reported by Nuñez *et al.* (1990). A nucleotide-nucleoside mixture given to rats improved the mucosal growth and maturity by increasing the proliferating activity of crypt cells (Tsujinaka *et al.*, 1993). Tanaka *et al.* (1996) suggested that exogenous nucleotides may have an important role in controlling the dynamic balance of cellular turnover in the developing small intestine of infants.

Experiments with rats (Ortega *et al.*, 1995a) supported the idea that dietary nucleotides affect the maturation status of small-intestine epithelium. Similarly nucleotide deprivation led to a decrease in fractional protein synthesis and DNA concentration in the intestine, suggesting that dietary nucleotides may modulate protein synthesis in the intestine (López-Navarro *et al.*, 1996b).

All the above findings from *in vivo* studies have been complemented with those from *in vitro* studies using different intestinal cell lines. Trials involving rat intestinal cell lines (IEC-6) and human colon carcinoma cell lines (Caco-2) showed that a nucleotide supplement supported the proliferation and differentiation of IEC-6 cultured under optimal and sub-optimal (nutritional stress) conditions, while growth of Caco-2 was supported only under conditions of nutritional stress (He *et al.*, 1993). These findings were also confirmed by Sato *et al.* (1999; 2000) in *in vitro* (Caco-2 and IEC-6) and *in vivo* experiments. Recently, the importance of nucleotides when nutrition is limited was further confirmed since exogenous RNA, DNA, mixtures of nucleotides or any deoxy mononucleotide (except TMP – thymine monophosphate) supported the growth of IEC-6 under nutritional stress conditions (Holen & Jonsson, 2004).

Response to injury

Nucleotides also have a protective role in the intestinal system. Dietary nucleosides were beneficial following radiation-induced intestinal injury in rats (Quan *et al.*, 1991). Nucleotide supplementation has also been reported to be beneficial against diarrhoeal disease in infants (Brunser *et al.*, 1994; Yau *et al.*, 2003) and rats (Norton *et al.*, 2001). It was confirmed by Bueno *et al.* (1994) that the intestinal histology and ultrastructure of rats suffering from diarrhoea was closer to the normal control group when the animals received a nucleotide-enriched diet. Ortega *et al.* (1995b) suggested that dietary nucleotide intake in the elderly may accelerate the normal physiological intestinal response to refeeding after food deprivation.

Total parenteral nutrition (TPN) consists of the intravenous administration of all the required nutrients when these cannot be adequately absorbed through the intestines. Dietary nucleic acid

supplementation is essential in patients under TPN (Tsujinaka *et al.*, 1993; 1997; 1999). Similarly Ogita *et al.* (2004) confirmed that nucleotides and nucleosides are essential for intestinal growth and maintenance of structures in small bowel transplantation.

1.4.4.2 Nucleotides and lipids

Many studies in infants and other mammals have reported the effects of dietary nucleotides on lipid metabolism and lipoprotein synthesis and secretion. This is of great importance during the early stages of life since lipids are the main structural components of cell membranes with effects on growth and development. However, no studies on similar effects have been conducted in fish despite the fact that relatively high amounts of lipids are mobilised from the liver into the fish gonads during oogenesis.

Lipid metabolism

Nucleotides are known to affect lipid metabolism in humans and other mammals (reviewed Carver & Walker, 1995). Ramirez *et al.* (1991) suggested that dietary nucleotides might modulate polyunsaturated fatty acid (PUFA) conversion and eicosanoid synthesis, since increased levels of arachidonic acid (ARA: 20:4n-6), prostacyclin (PGI2) and thromboxane (TXA2) were found in the plasma of rats fed a nucleotide supplemented diet. Jimenez *et al.* (1992) showed that dietary nucleotides caused an increase in the n – 6 long chain PUFA, especially ARA, in the membrane of red blood cells of rats. Boza *et al.* (1992) studying plasma fatty acid levels in weanling rats also reported a significant increase in fatty acids, namely monounsaturated and poly-unsaturated fatty acids of the n – 3 and n – 6 series in the group fed the nucleotide diet, suggesting an important modulation of PUFA synthesis by dietary nucleotides. Nuñez *et al.* (1993) suggested that dietary nucleotides modify PUFA metabolism by lowering the delta-9 desaturase activity and increasing activities of delta-5 and delta-4 in the rat liver. Furthermore, in a study looking at the effects of dietary nucleotides on lipid metabolism and the learning ability of rats it was reported that the levels of docosahexaenoic

acid (DHA: 22:6n-3) and ARA in the phosphatidylcholine fraction of the cerebral cortex were increased in the group fed the nucleotide supplemented diet (Sato *et al.*, 1995). The administration of a nucleotide-supplemented diet to rats corrected the altered levels of saturated, monounsaturated and n – 6 long-chain polyunsaturated fatty acids in plasma and liver microsomes produced by induced liver cirrhosis (Fontana *et al.*, 1998; 1999).

However, other studies have reported that dietary nucleotides caused no changes in the profile of long-chain PUFA on red blood cells of preterm infants (Axelsson *et al.*, 1997), nor augmentation of the erythrocyte and plasma cholesterol ester long chain PUFA of low-birthweight babies (Woltil *et al.*, 1995).

Lipoproteins

Early studies using rats suggested that nucleotides affect liver lipoprotein secretion through changes in the synthesis of apolipoproteins (apo) (Windmuller, 1964; Windmuller & Levy, 1967). Nucleotide supplementation increased plasma lipoproteins in newborns. Newborn rats fed diets supplemented with nucleotides increased plasma very low density lipoproteins (VLDL) and high density lipoproteins (HDL) concentrations (Garcia-Molina *et al.*, 1991), while HDL-cholesterol was increased in newborn infants (Sánchez-Pozo *et al.*, 1986). In small-for-gestational-age (SGA) infants the group fed the nucleotide-supplemented formula had enhanced lipoprotein concentrations due to increased total apo concentrations in all lipoproteins as well as increased apo A-I in HDL and VLDL and apo B-100 in VLDL and low density lipoproteins (LDL) (Morillas *et al.*, 1994). Similarly, increased apo content was the cause of the increase in lipoprotein levels in the plasma of preterm infants fed nucleotide-enhanced formulas reported by Sánchez Pozo *et al.* (1994). Furthermore, nucleotides also increased plasma concentrations of apo A-IV which induced higher activity of plasma lecithin-cholesterol acyl transferase, a key enzyme in lipoprotein metabolism (Sánchez-Pozo *et al.*, 1995a). More recently, Siahanidou *et al.* (2004) reported preterm neonates fed from birth with a nucleotide-supplemented formula to have significantly higher HDL-cholesterol and lower LDL-cholesterol serum levels than those from the group fed unsupplemented formula.

1.4.4.3 Hepatic effects

The liver plays a major role in meeting somatic nucleotide requirements through active synthesis and release for use by other tissues. The hepatic supply of nucleotides is maintained through *de novo* synthesis and salvage pathways (Carver & Walker, 1995). However, results suggest that liver nucleotide metabolism is modulated by the availability of dietary nucleotides (López-Navarro *et al.*, 1995).

Hepatic morphology and proliferation

Novak *et al.* (1994) reported that the liver weight (expressed as a percentage of body weight), and glycogen were higher in animals fed nucleotide diets versus controls. Furthermore, later studies showed that the restriction of dietary nucleotides in animals may result in hepatic lipid accumulation and decreased mucosal height and gut wall thickness (Carver, 1994). Dietary nucleotide deficiency can alter nucleotide levels and have adverse effects on cell proliferation (Jackson *et al.*, 1995), and also affect liver ultrastructure and function by a reduction of the hepatocyte nuclear and nucleolar areas. It can also affect nuclear chromatin condensation, reduce the rough endoplasmic reticulum, ribosome association and abundance, and produces accumulation of fat (López-Navarro *et al.*, 1996a). The nucleotide-deprived group also presented lower RNA concentrations and fractional protein synthesis rates in the liver (López-Navarro *et al.*, 1996b). Liver RNA levels were also reported to decrease by deprivation of dietary nucleotides and starvation in young and adult rats (López-Navarro *et al.*, 1997).

Dietary nucleotides can also modulate gene expression by interaction with specific transcription factors, in both the liver and the small intestine (Sánchez-Pozo & Gil, 2002). The phenomenon of exogenous nucleosides enhancing the proliferation of hepatic cells at gene expression level was also reported by Saez-Lara *et al.* (2004).

Response to injury

The damage resulting from thioacetamide intake increases liver collagen, but dietary nucleotides are known to prevent excessive hepatic deposition of this protein (Torres *et al.*, 1997a). This reduction of hepatic fibrosis in nucleotide-supplemented rats was suggested to rely on the enhancement of collagenase activity and the reduction of collagen content and maturation by the nucleotides (Perez *et al.*, 2002). Cirrhotic rats that consumed a semipurified diet supplemented with nucleotides for two weeks showed considerable histological regeneration of the injured liver (Torres-López *et al.*, 1996; Torres *et al.*, 1997b). Furthermore, the administration of nucleoside-nucleotide mixtures improved hepatic regeneration in *in vitro* and *in vivo* studies using partially-hepatectomized rats (Usami & Saitoh, 1997).

Different studies have confirmed that the alterations of fatty acid levels and lipid metabolism in cirrhotic livers can be corrected by using dietary nucleotides (Fontana *et al.*, 1998; 1999; Leite *et al.*, 2000). Furthermore, the protective effect of nucleotides on hepatic injury was also reported by Norton *et al.* (2001) in rats whereas Yokoyama *et al.* (2004) demonstrated the anti-carcinogenic effects of nucleotides against liver tumors in mice.

1.4.4.4 Immunological effects

Evidence from many animal and human studies supports the idea that dietary nucleotides are important for the optimal function of the immune system. The effects of nucleotides on the immune system have been reviewed several times (e.g. Carver & Walker, 1995; Maldonaldo *et al.*, 2001). Mice fed a nucleotide-free diet were reported to have impaired cellular and humoral immune responses (Carver, 1994; Jyonouchi *et al.*, 1994; Kulkarni *et al.*, 1994). One of the mechanisms by which a nucleotide-free diet suppresses the immune response is by blocking the maturation of T-lymphocytes. However, the addition of RNA to the same formula restores maturation again (Kulkarni *et al.*, 1994).

Nucleotides are known to facilitate macrophage phagocytosis and resistance to bacterial challenge (Kulkarni *et al.*, 1986a,b) and also increase natural killer cell (NK) activity. Carver *et al.* (1994) found that NK cytotoxicity in 2-month old infants fed a nucleotide-supplemented formula (NT+) was significantly higher compared to the control group (NT-). Interleukin-2 production by stimulated mononuclear cells was also higher in the NT+ compared with the NT- group. Nucleotides also promote the recovery of the depressed immune system after a period of malnutrition or starvation (Pizzini *et al.*, 1990).

Pickering *et al.* (1998) described how an infant formula fortified with nucleotides enhanced *Haemophilus influenzae* type b and diphtheria humoral antibody responses. The stimulation of T lymphocytes by dietary nucleotides was also reported by Nagafuchi *et al.* (1997) in mice. Nucleotides also enhanced immunoglobulin (Ig) A and IgM in preterm infants, suggesting that dietary NT may affect Ig production in immunocompromised children (MartinezAugustin *et al.*, 1997; Navarro *et al.*, 1999).

In summary, nucleotides promote lymphocyte maturation and proliferation, macrophage phagocytic activity, Ig production, defence against infections and also modulates the hypersensitivity reaction after organ transplantation (reviewed Gil, 2002). Since human milk is known to contain higher levels of nucleotides than cow milk, Uauy *et al.* (1994) first suggested that "*infant formulas should have sources of nucleotides added to duplicate human milk and provide these substrates for maximal intestinal development and repair*". Based on all the evidence that exogenous nucleotides have important positive effects for the development of immune and gastrointestinal systems in infants the European Union recognised nucleotides as semi-essential components and prepared norms to regulate these compounds in baby formulas (Commission Directive 96/4/EC).

A new application has been suggested recently. Since microgravity provokes adverse effects on health, with loss of bone and muscular mass and depletion of the immune system among others, nucleotides may help to reduce this immunosupression. In an experiment with mice, nucleotides

were successfully used as immunostimulants to maintain and restore the immune response during microgravity conditions (Kulkarni *et al.*, 2002; Yamauchi *et al.*, 2002). Therefore, these compounds are now suggested as nutritional supplements for NASA astronauts.

1.4.4.5 Nucleotide-enhanced diets and fish

The first studies reporting the use of nucleotides in fish diets are from the 1980's when Tacon & Cooke (1980) used practical diets supplemented with bacterial-RNA to feed trout (*Oncorhynchus mykiss*). It was reported that RNA supplementation had negative effects on growth rate and feed intake in trout. Studies on rainbow trout (*Oncorhynchus mykiss*) described that increasing levels of dietary yeast-RNA increased growth rate, feed efficiency and carcass nitrogen retention however, dietary free base adenine was reported as a potent inhibitor of feed intake and growth (Rumsey *et al.*, 1992). Similarly, diets enhanced with ASCOGEN ®, a nucleotide supplement, increased growth rates in European catfish (*Silurus glanis*) and rainbow trout (Hamackova *et al.*, 1992; Adamek, 1994; Adamek *et al.*, 1996). Growth increase and improved survival were also reported in seabream (*Sparus aurata*) larvae fed dietary nucleotides (Borda *et al.*, 2003). More recently the use of dietary ribonucleic acid (RNA) as a source of exogenous nucleotides was tested in sea bass (*Dicentrarchus labrax*) (Peres & Oliva-Teles, 2003) and it was reported that RNA extracts promoted an increase in urinary and branchial nitrogen excretion. However, the final body weight and specific growth rate were decreased when the levels of RNA in the diet were too high (12.4%); possibly due to an insufficient capacity of fish uricase to degrade nucleic acid into urea (Oliva-Teles *et al.*, 2003).

Ishida & Hidaka (1987) reported the use of nucleotides as feed enhancers for several marine teleosts. This was later confirmed by Ikeda *et al.* (1991) in jack mackerel (*Trachurus japonicus*) and by Kubitza *et al.* (1997) in largemouth bass (*Micropterus salmoides*). However, most of the studies involving nucleotides and fish aimed to test these compounds as immunostimulants. Nucleotide supplemented diets were first reported to produce an elevated immune response in

hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureaus*). Both B-cell and T-cell activities were increased in tilapia fish vaccinated against *Aeromonas hydrophila* (Ramadan *et al.*, 1994). The enhancement of the non-specific immune system in carp (*Cyprinus carpio*) fed nucleotides from yeast RNA was reported by Sakai *et al.* (2001). The phagocytic activity of the kidney leucocytes, nitroblue tetrazolium (NBT) activity – used to measure neutrophil oxidative radical production –, lysozyme activity and complement activity were significantly higher in fish from the nucleotide group. The disease resistance of rainbow trout, Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) against bacterial, viral, rickettsial and ectoparasitic infections was increased with the supplementation of commercial salmonid diets with additional exogenous nucleotides (Burrells *et al.*, 2001a); nevertheless, an enhancement of macrophage killing capacity, as reported in tilapia (Ramadan *et al.*, 1994), was not demonstrated in that study. Elevated antibody and lower mortality of Atlantic salmon after challenge with *Aeromonas salmonicida* demonstrated that nucleotides enhanced the efficacy of vaccination (Burrells *et al.*, 2001b). In turbot (*Scophthalmus maximus*) the supplementation of a commercial diet with nucleotides exerted a consistent effect on the humoral specific immune response at gene expression level in different lymphoid tissues. The IgM gene and RAG-1 expression were significantly higher in the gills and spleen of turbot fed the nucleotide diet (Low *et al.*, 2003).

Dietary nucleotides have also been effective against sea lice and Atlantic salmon fed a NT-supplemented diet had significantly less lice attached and significantly lower re-infestation rates following bath treatment with EXCIS™ compared to the control group (Burrells *et al.*, 2003). Furthermore, juvenile rainbow trout that were fed with a nucleotide diet showed higher indices of B and T lymphocytes and normal cortisol levels in plasma. When the fish were infected with infectious pancreatic necrosis virus (IPNV), the nucleotide diet was able to stimulate the production of B lymphocytes and decrease plasma cortisol levels. All the fish infected with IPNV and fed the normal diet died after one week, while all the animals from the nucleotide-fed group survived (Leonardi *et al.*, 2003). The effects of nucleotide-supplemented diets (ASCOGEN-P ®) on the immune response of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) were also investigated by Li *et al.* (2004). The neutrophil oxidative radical production

and the antibody response based on microtitration agglutination were significantly higher in fish fed the nucleotide-supplemented diet, which also exhibited significantly enhanced survival after exposure to *Streptococcus iniae.*

Other important physiological effects were reported by Burrells *et al.* (2001b) regarding significantly reduced blood chloride levels following salt water transfer of Atlantic salmon, indicating a greatly enhanced capacity for osmoregulation in the group fed the nucleotide-enriched diet. Furthermore, improvements in fold morphology and the number of villi in the gut have been reported for adult salmon (Burrells *et al.*, 2001b) and seabream larvae (Borda *et al.*, 2003) fed nucleotide-enhanced diets (Table 1. 3).

Table 1. 3 Studies reporting the use of nucleotides in fish diets.

Topic	Name	*Species*	Reference
Growth performance	Rainbow trout	*Oncorhynchus mykiss*	Tacon & Cooke, 1980 Rumsey *et al.*, 1992 Adamek, 1994 Adamek *et al.*, 1996
	European catfish	*Silurus glanis*	Hamackova *et al.*, 1992 Adamek *et al.*, 1996
	Seabass	*Dicentrarchus labrax*	Peres & Oliva-Teles, 2003
	Seabream	*Sparus auratus*	Borda *et al.*, 2003 Oliva-Teles *et al.*, 2003
Feed enhancer	Rabbitfish	*Siganus fuscescens*	Ishida & Hidaka, 1980
	Chicken Grunt	*Parapristipoma trilineatum*	Ishida & Hidaka, 1980
	Greater amberjack	*Seriola dumerili*	Ishida & Hidaka, 1980
	Jack mackerel	*Trachurus japonicus*	Ishida & Hidaka, 1980 Ikeda *et al.*, 1991
	Chub mackerel	*Scomber japonicus*	Ishida & Hidaka, 1980
Immune system	Hybrid tilapia	*Oreochromis niloticus* X *O. aureaus*	Ramadan *et al.*, 1994
	Carp	*Cyprinus carpio*	Sakai *et al.*, 2001
	Atlantic salmon	*Salmo salar*	Burrells *et al.*, 2001a,b Burrells *et al.*, 2003
	Coho salmon	*Oncorhynchus kisutch*	Burrells *et al.*, 2001a
	Rainbow trout	*Oncorhynchus mykiss*	Burrells *et al.*, 2001a Leonardi *et al.* 2003
	Turbot	*Scophthalmus maximus*	Low *et al.*, 2003
	Hybrid stripped bass	*Morone chrysops* X *M. saxatilis*	Li *et al.*, 2004
Osmoregulation	Atlantic salmon	*Salmo salar*	Burrells *et al.*, 2001b
Gut	Atlantic salmon	*Salmo salar*	Burrells *et al.*, 2001b
	Seabream	*Sparus aurata*	Borda *et al.*, 2003

As explained above, oogenesis is a process of intense cell division, with high DNA and RNA formation and hence also a high requirement for nucleotides. The effects that an exogenous supply of nucleotides may have on this process and also reproduction have never been investigated. This project tested the efficacy of using nucleotide-enhanced diets on fish diets and studied their effects on fish reproduction of Atlantic halibut and haddock. The following hypotheses will be addressed through the different sections of the thesis:

- The nucleotide-enhanced diets affect the spawning performance of fish and the condition of the broodfish after the spawning season.
- Egg quality is improved through the supplementation of broodstock diets with extra nucleotides.
- Dietary nucleotides given to the broodstock affects also larval quality
- The egg nucleotide composition will be altered as a result of the different broodstock dietary treatments.
- Furthermore, energy charge in eggs will be increased by the nucleotide-enriched broodstock diet.
- As for carp (Boulekbache *et al.*, 1989), adenine energy charge is a indicator of egg quality and larval quality in halibut and haddock.

Chapter 2: Broodstock nutrition

2.1 Introduction

2.1.1 Broodstock nutrition

Although fish nutrition research has developed rapidly in the last twenty years, especially due to the impulse and development of aquaculture, there are many areas that need further research. Broodstock nutrition is without doubt one of the most important, but also poorly understood areas of fish farming. Experiments involving broodfish are time-consuming and expensive due to the need for suitable facilities for maintaining large groups of adult fish. Most of the known dietary requirements of fish are derived from studies on juvenile and relatively fast-growing fish. However, reproduction is a completely different process to that of growth and imposes a different and additional metabolic burden; therefore, the specific nutritional requirements of broodfish are different when compared to those of fast growing juveniles. For example, while essential fatty acid requirements for sparid juveniles range between 0.5 - 0.8 % n – 3 HUFA (highly unsaturated fatty acids) in the diet (Izquierdo, 1996), those determined for sparid broodstock ranges between 1.5 - 2 % n – 3 HUFA in the diet (Watanabe *et al.*, 1984a, d, e; 1985; Fernández-Palacios *et al.*, 1995). Furthermore, dietary antioxidant requirements increase during reproduction (Izquierdo & Fernández-Palacios, 1997).

In the last 20 years, the number of studies in this area has increased. However, information about the more specific nutritional needs of maturing fish is still sparse when compared to those at other developmental stages. Broodstock nutrition studies were pioneered in the early 1980's by Toshio Takeuchi and Takeshi Watanabe using rainbow trout and red seabream. It was demonstrated that low protein-high calorie diets supplemented with beef tallow had no adverse effects on reproduction of rainbow trout, and that a trace element supplementation to fish meal

diets was indispensable for reproduction (Takeuchi *et al.*, 1981). Among all the possible factors which affect gonadal maturation and spawning, the quality of the feed was found to be closely related to the reproduction of red seabream (Watanabe *et al.*, 1984a) and rainbow trout (Watanabe *et al.*, 1984c). The results demonstrated that reproduction and egg quality were deeply affected by the nutritional quality of diets given to broodstock.

In addition, several studies have shown that quantity of the diet is also important in broodstock feeding and reproduction. Studies on rainbow trout found that while all fish fed on full rations spawned, 11% of the group fed half rations failed to mature (Springate *et al.*, 1985). Fecundity, egg size and dry weight, serum calcium (as an index of vitellogenin) in females and testosterone levels in males were all significantly higher in the broodstock fed on full ration when compared to the half-ration group. A reduction in the feeding rate has been reported to affect and even inhibit gonadal maturation in many species such as haddock (*Melanogrammus aeglefinus*) (Hislop *et al.* 1978), winter flounder (*Pseudopleuronectes americanus*) (Tyler & Dunn 1976), goldfish (*Carassius auratus*) (Sasayama & Takahashi, 1972), European seabass (*Dicentrachus labrax*) (Cerda *et al.*, 1994a) and Atlantic salmon (*S. salar*). This failure in gonadal maturation has been associated with endocrine disruptions as a result of the diet. Thus, detrimental effects of food deprivation were also associated with reduced levels of plasma estradiol in female seabass (Cerda *et al.*, 1994a).

However, most of the nutritional studies carried out on broodstock have focussed not on the feed ration but on the quality of the feeds by supplementing/replacing different compounds in the diet. Increasing attention has been paid to the role of different dietary components in the last 10 years, demonstrating that nutrition affects many aspects of fish reproduction. Navas *et al.* (1998) reported that differences in the diet composition between experimental and natural diets produced changes in the plasma levels of 17β – estradiol, vitellogenin, gonadotropin II, and altered the spawning performance of seabass.

Effects of nutrition on broodfish fecundity

Fecundity is defined as the number of eggs produced per fish. Egg production by fish is determined by fish size, thus under normal conditions bigger fish will produce more eggs. Therefore, when comparing egg production in broodstock studies it is essential to consider fish size. Relative fecundity is defined as the number of eggs produced per fish weight and is expressed as number of eggs / unit body weight. Relative fecundity, called fecundity from here onwards, has been reported to be affected by broodstock diet: fecundity in red seabream was found to increase as a result of the diet. Diets in which fish meal was substituted for cuttlefish meal significantly improved broodstock fecundity (Watanabe *et al.*, 1984b, d, e). Duray *et al.* (1994) reported an increase in the fecundity of rabbitfish (*Siganus guttatus*) when dietary lipid levels in the broodstock diet were elevated from 12 % to 18 %. Fecundity was also significantly improved when increasing n –3 HUFA levels from 1.3 % up to 1.6 % (Fernández-Palacios *et al.*, 1995) and when giving squid meal to gilthead seabream (*Sparus aurata*) (Fernández-Palacios *et al.*, 1997). A broodstock diet including squid meal and astaxanthine in a dry pellet improved fecundity and egg quality in striped jack (*Pseudocaranx dentex*) up to the levels of those achieved with the traditional raw fish-based diet (Vassallo-Agius *et al.*, 2001b). Recently, as with larval studies, much research has focused on the role of essential fatty acids (EFA) in the reproduction of fish. An n – 3 HUFA deficiency in the broodstock diet has a negative effect on gilthead seabream fecundity (Almansa *et al.*, 1999), in contrast to an improvement in rainbow trout (Vassallo-Agius *et al.*, 2001a). Increasing n – 3 HUFA in the diet of Japanese flounder (*Paralichthys olivaceus*), '*tended to increase fecundity though these results were not statistically significant*' (Furuita *et al.*, 2000; 2002). The importance of n – 6 HUFA in reproduction, and in particular arachidonic acid (ARA, 20:4n – 6) has also been established since ARA is the major eicosanoid precursor in fish cells (Tocher & Sargent, 1987; Bell *et al.*, 1994). Arachidonic acid and these compounds are important in the control of ovulation (Sorbera *et al.*, 2001). An ARA-enhanced broodstock diet was found to increase relative fecundity in Atlantic halibut (*Hippoglossus hipploglossus*) (Mazorra *et al.*, 2003). Alorend *et al.* (2003) with Atlantic halibut and Furuita *et al.* (2003b) with Japanese flounder also reported increases in

fecundity up to a certain level of ARA in the diet. However, very high levels were found to have detrimental effects.

Other nutrients which have been shown to affect fecundity include vitamin A in Japanese flounder (Furuita *et al.*, 2003a), vitamin E in gilthead seabream (Izquierdo & Fernández-Palacios, 1997), and vitamin C in rainbow trout (Blom & Dabrowski, 1995). The effect of carotenoid supplementation in broodstock diets has also been studied. Watanabe *et al.* (1984e) reported that addition of 0.1 and 0.3 % of β-carotene and canthanxanthin in the diet produced significantly lower numbers of eggs than a control diet with a high protein content although the quality of the eggs and larvae was better. Inclusion of astaxanthin was reported to increase fecundity in yellowtail broodstock (Verakunpiriya *et al.*, 1997) and also in striped jack (Vassallo-Agius *et al.*, 2001b, c). Possible effects of dietary vitamin C on rainbow trout oogenesis as a result of increased serum levels of vitellogenin and 17β – estradiol were reported by (Waagbø *et al.*, 1989). An extensive review on ascorbic acid and fish reproduction was recently undertaken (Dabrowski & Ciereszko, 2001).

Effects of broodstock nutrition on fertilisation

Fertilisation is one of several parameters that can be used to assess egg quality in fish. Several authors have reported on its improvement associated with broodstock diet. Fertilisation rates were increased in gilthead seabream and turbot broodstock fed increased levels of n – 3 HUFA (Fernández-Palacios *et al.*, 1995; Lavens *et al.*, 1999) and also in striped jack fed with squid meal (Vassallo-Agius *et al.*, 2001b, d). An n – 3 HUFA deficiency in the diet of gilthead seabream produced a reduction of this parameter (Almansa *et al.* 1999) while Fernández-Palacios *et al.* (1995; 1997) found a clear positive correlation between dietary levels of eicosapentanoic acid (EPA: 20:5n – 3) and ARA on fertilisation rates in the same species. Mazorra *et al.* (2003) also found that fertilisation was significantly improved when Atlantic halibut was fed an ARA-enriched diet. Furthermore, Asturiano *et al.* (2001) discovered that the reproductive performance of male seabass was improved when fish were given diets enriched with essential polyunsaturated fatty acids (PUFA): the spermiation period was longer, and milt

production, spermatozoa density, egg development and larval survival was significantly higher with the PUFA diets. It is possible that improvements in fertilisation are a reflection of enhanced sperm performance.

Apart from EFA, other important nutrients for fertilization are vitamins. An increase in α-tocopherol levels in gilthead seabream was associated with higher fertility and larval survival (Izquierdo and Fernández-Palacios, 1997). As mentioned above, vitamin C is known to be important for fish reproduction and it has been widely studied especially in rainbow trout (e.g. Dabrowski & Ciereszko, 2001). An ascorbate deficiency significantly reduced sperm concentration, total sperm production over the season and sperm motility (Ciereszko & Dabrowski, 1995). Ascorbic acid levels in seminal plasma reflected the dietary intake of this nutrient (Dabrowski & Ciereszko, 1996). When studying the motility and fertilising ability of rainbow trout fed diets without ascorbic acid and enhanced with ascorbyl monophosphate, the findings revealed that the spermatozoa from the supplemented group had the highest motility and lowest decline in fertilizing ability and suggested that the positive effects of this vitamin on milt quality was related to long-term effects during spermatogenesis (Ciereszko & Dabrowski, 2000).

Effects of broodstock nutrition on embryo development

The normal development of fish embryos is typically assessed through the morphology of the eggs and the hatching rates. Equal blastomeres with symmetrical early cleavages, transparency, distribution of lipid droplets, size of perivitelline space and changes in egg diameter after fertilisation are all morphological parameters associated with egg quality (see Kjørsvik *et al.*, 1990). More details on egg quality can be found in Chapter 3.

Energy reserves in fish eggs are finite and constitute the source of metabolic energy needed throughout embryonic development. Yolk composition in fish eggs and yolk sacs depends upon maternal supply and embryo and larvae depend on it until exogenous feeding starts. Takeuchi *et al.* (1981) first reported no detrimental effects on embryo development and hatching when red

seabream were fed a low protein-high calorie diet supplemented with beef tallow. However Cerda *et al.* (1994b) found the opposite in seabass, mirroring the results from Watanabe *et al.* (1984a) in which a low protein diet compromised egg production and the percentage of floating eggs; increased the percentage of abnormal eggs (e.g. higher number of lipid droplets) and deformed larvae, and significantly reduced hatching rates, larval growth and survival. In the same study, similar effects were found with those broodfish fed phosphorous or EFA-deficient diets, while an improvement in all the parameters studied was observed when cuttlefish meal was used instead of white fish meal (Watanabe *et al.*, 1984a, b, e; 1991b). Similarly the use of frozen raw krill in the same species led to a significant improvement in egg quality comparable to those from the previous experiments using cuttlefish meal. Later investigations (Watanabe *et al.*, 1991a; b) showed that both polar and non-polar lipid fractions contain important nutritional components for red seabream broodstock. Phosphatidyl choline and astaxanthin from the polar and non-polar fractions were attributed this positive effect. Increasing n – 3 HUFA dietary levels significantly improved the percentage of morphologically normal eggs in gilthead seabream (Fernández-Palacios *et al.*, 1995), whereas the number of lipid droplets in eggs was higher with a deficient diet (Fernández-Palacios *et al.*, 1997), as with red seabream. Pickova *et al.* (1997) also noted the importance of docosahexanoic acid (DHA: 22:6n – 3), EPA and ARA since the DHA/EPA ratio and ARA in the phospholipid fraction are positively correlated with egg symmetry and viability. The importance of feeding broodstock with correct DHA/EPA/ARA ratios was proved by Bruce *et al.* (1999) and Navas *et al.* (2001) reported that higher hatching rates in seabass eggs were associated with higher DHA:EPA and ARA:EPA ratios, with fatty acid composition of eggs affected by the diet. Similarly, inclusion of high levels of n – 3 HUFA into Japanese flounder broodstock diet decreased the egg quality by reducing ARA in the eggs (Furuita *et al.*, 2002). This was later confirmed by Mazorra *et al.* (2003) when higher levels of ARA improved blastomere morphology index and hatchability of Atlantic halibut eggs and by Furuita *et al.* (2003b) who reported improved Japanese flounder egg and larval quality with ARA up to 0.6%; detrimental effects occurring with higher levels.

With regard to vitamins and carotenoids, Sandnes *et al.* (1984) found no influence on the egg size of rainbow trout fed with different ascorbic acid levels. However, the vitamin C deficient diet showed significantly lower egg survival and hatching rates when compared to the non-deficient groups. This vitamin is known to be necessary for the synthesis of collagen during embryo development. The addition of α – tocopherol (200 mg/100 g) in red seabream diet improved egg and larval quality (Watanabe *et al.*, 1991a; b). Fernández-Palacios *et al.* (1997) also found that egg viability and percentage of abnormal eggs improved with increasing levels of α – tocopherol. Recently, the beneficial effects of vitamin A-supplementation on the egg quality of Japanese flounder were reported by Furuita *et al.*(2001; 2003a). Carotenoid supplementation improved egg quality for red seabream (Watanabe & Kiron, 1995) and yellowtail (Verakunpiriya *et al.*, 1996) but did not improve egg quality in striped jack since the compound was not incorporated into their eggs (Vassallo-Agius *et al.*, 1998).

Effects of broodstock nutrition on larval quality

The number of studies reporting effects of broodstock nutrition on larvae are fewer than those on egg quality. Watanabe *et al.* (1984a) first reported the effects that dietary protein for red seabream broodstock had on larval quality. Low protein diets given to broodstock resulted in lower fry survival and 96% deformity, while larval quality was improved when cuttlefish meal added to the diet (Watanabe *et al.* 1984a, b, e; 1991b). The use of frozen raw krill in the diet resulted in growth and swim bladder inflation rates comparable to those from the cuttlefish meal trial (Watanabe *et al.*, 1984e). In these experiments, the effect of dietary EFA on larval quality was also studied and the use of corn oil (rich in linoleic acid: 18:2n – 6) instead of fish oil reduced egg and larval quality. Increasing dietary levels of n – 3 HUFA above 1.6 % in the broodstock diet significantly reduced survival of gilthead seabream larvae (Fernández-Palacios *et al.*, 1995). The use of broodstock dietary regimes that increase the DHA/EPA and ARA/EPA ratio in eggs and therefore improve larval quality and survival was suggested by Bell *et al,* (1997). In the same year, Navas *et al.* (1997) identified vitellogenesis as the period of ovarian maturation during which dietary EFA are incorporated most effectively into the developing oocytes. A significant increase in the percentage of normal larvae and survival activity index

(SAI: calculated from the number of surviving larvae and their survival duration) was reported when Japanese flounder were fed increasing levels of n – 3 HUFA (Furuita *et al.*, 2000) although extremely high levels reduced these parameters (Furuita *et al.* 2002).

Tilapia (*Oreochromis mossambicus*) broodstock fed an ascorbic acid-supplemented diet improved hatchability and larval condition, while those fed a non-supplemented diet produced deformed fry with poor growth, food utilisation and survival rate (Soliman *et al.*, 1986). Vitamin A also reduced the number of deformed larvae in Japanese flounder (Furuita *et al.*, 2003a). Using different levels of astaxanthin, 30 ppm in the diet of yellowtail broodstock produced the highest quality larvae (Verakunpiriya *et al.*, 1997). Furthermore, lack of minerals such as phosphorus in the broodstock diet led to extremely high deformity levels in red seabream larvae together with depleted growth and survival rates (Watanabe *et al.*, 1984a).

In summary, although the number of studies on broodstock nutrition has increased in the last ten years, the information regarding nutrient requirements is limited to a few species. The nutritional needs of broodfish are distinct to those of juveniles and in many cases are species dependent. Deficiency, excess or imbalanced amounts of nutrients will lead to detrimental effects on reproduction; more information is required. Furthermore, the timing of broodstock nutrition varies with species and their vitellogenic periods. In fish with short vitellogenic periods, such as red seabream and gilthead seabream, egg composition is readily affected by the diet within a few weeks of feeding and the spawning can be improved by using broodstock diets that cover their nutritional needs even during the spawning season. Conversely, batch spawners with long vitellogenic periods need to be fed with enhanced feeds for several months before the spawning season in order to have an effect on egg composition.

Up until now, no studies have looked at the effectiveness of nucleotide supplementation of broodstock feeds on any parameter involved in fish reproduction and most published works used juvenile fish to investigate effects on the immune system and growth performance.

Fecundity can be affected by ovarian atresia, so this phenomenon was also studied in the present project.

2.1.2 Ovarian follicular atresia

Follicular atresia refers to those processes during which the ovarian follicle loses its integrity and the egg is disposed of by means other than ovulation. It is a highly regulated degenerative process in the ovary of all vertebrates and believed to be essential for the maintenance of ovarian homeostasis (Wood & Van Der Kraak, 2001). In teleosts, it is observed in all stages of the reproductive cycle although most frequently during the post-spawning period. A number of investigators have described atresia in teleost ovaries (e.g. Kjesbu *et al.*, 1991; Miranda *et al.*, 1999; Ganias *et al.*, 2003). Atresia is characterised by (Figure 2. 1):

- The disintegration of the nucleus, vitelline envelope breakdown and an increase in the number and size of follicular (granulosa) cells.
- Liquefaction of yolk globules with follicular cells entering the oocytes to phagocytise degenerating material.
- Degeneration of the follicular cells once yolk resorption is complete and eventually fibroblast-like cells around yellowish-brown material (lipofuscin/ceroid) remain.

In mammals the majority (75%-99.9%) of the finite pool of follicle-enclosed oocytes are eliminated before ovulation and become atretic (Byskov, 1978). Teleosts however have a higher fecundity than other vertebrates and recruit many thousands of oocytes into the ovulatory pool during each spawning season.

Figure 2. 1 Different levels of atresia in postvitellogenic oocytes of haddock. Previtellogenic oocytes, much smaller in size, are also present (right bottom corner).

Ovarian follicular atresia is common in vertebrate ovaries under both natural and experimental conditions. In teleost fish, it can be induced by factors such as stress, fasting, biocides, light, temperature, confinement and inadequate hormone levels (Nagahama, 1983). Since it is a degenerative process occurring in the ovary, atresia affects fecundity. In this study, all the remaining haddock females at the end of the second spawning season were culled and ovarian samples were collected in order to study the atresia levels (§ 2.3.5).

2.2 Aims

The main aim of this part of the project was to study potential differences in the spawning performances of broodstock fed the control and nucleotide-enriched diets. Additionally, broodstock condition at the end of each spawning season was also compared between diets. Gonadosomatic and hepatosomatic indices were also studied and fish gonads were examined histologically to assess the atresia levels.

2.3 Material and Methods

As explained in chapter 1, Atlantic halibut (*Hippoglossus hippoglossus,* L.) was used during the 2001 spawning season, while haddock (*Melanogrammus aeglefinus*, L.) was used for two consecutive spawning seasons, 2002 and 2003. Information related to broodstock details, gamete collection and feeding regimes for each species are discussed in this section.

2.3.1 Broodstock

Atlantic halibut and haddock husbandry was carried out at SAMS marine laboratory at Ardtoe, formerly Seafish Ardtoe. Six broodstock tanks (4 x 20 m^3, 2 x 15 m^3) were set up. The seawater was pumped from Ardtoe Sound at 6 m depth; UV treated (250 mV) and passed through sand filtration (15 μm). Flow rates were set to 25 $L \cdot min^{-1}$, salinity was > 32 ‰ and oxygen was > 7 $mg \cdot L^{-1}$. The photoperiod was ambient for the West Coast of Scotland.

2.3.1.1 Atlantic halibut broodstock

Broodstock resources consisted of six groups of fish each containing five male and five female Ardtoe hatchery-reared Atlantic halibut. There were sixty 7-year-old fish in total with an average body weight of 8 kg. All were PIT (Passive Integrated Transponder)-tagged next to the dorsal fin for individual identification, although visual identification of individual fish based on physical characteristics was also possible (Table 2. 1).

Fish were transferred to the experimental broodstock tanks (10 fish per tank -5 ♂: 5♀-) six weeks before the nucleotide-enriched diet was first given. A prophylactic formalin treatment was conducted prior to the start of the trial to remove any ectoparasites. Since chilling of broodstock water supplies has beneficial effects on quantity and viability of eggs spawned

(Brown *et al.*, 1995), water was chilled to provide a suitable temperature profile for halibut spawning (6 °C) and recorded daily.

From the six groups of ten animals three were fed a nucleotide-enriched diet, whilst the remainder were fed with the control diet. All fish had been fed on the control diet before the trial started, during the acclimation period. The test diet was first introduced on 21st October 2000 and the trial finished on 30th May 2001.

Table 2. 1 Halibut female broodstock and spawning information at the end of the spawning season (30/05/2001).

Diet	Tank	Female code	PIT No.	Length (m)	Weight (kg)	Spawned	No. of strippings	Female mean weight (kg)	Spawning female mean weight (kg)
Control	F1	F1-1	127	0.90	10.2	YES	5	8.4 ± 0.66	8.07 ± 0.73
		F1-2	363	0.80	7.9	YES	4		
		F1-3	801	0.80	7.0	YES	1		
		F1-4	608	0.87	9.7	NO	0		
		F1-5	298	0.82	7.2	YES	2		
Nucleotide	F2	F2-1	585	0.77	6.2	YES	1	7.7 ± 0.82	7.4 ± 1.00
		F2-2	807	0.85	9.2	YES	0		
		F2-3	845	0.86	8.7	NO	0		
		F2-4	311	0.90	5.2	YES	5		
		F2-5	098	0.81	9.0	YES	4		
Control	F3	F3-1	066	0.86	7.7	YES	2	8.1 ± 0.8	8.5 ± 0.86
		F3-2	379	0.94	10.8	YES	3		
		F3-3	026	0.83	6.3	NO	0		
		F3-4	297	0.80	8.7	YES	3		
		F3-5	014	0.86	6.8	YES	2		
Nucleotide	R1	R1-1	533	1.00	13.2	YES	5	9.46 ± 1.92	9.46 ± 1.92
		R1-2	300	0.76	5.8	YES	0		
		R1-3	578	0.74	6.8	YES	7		
		R1-4	890	0.80	6.5	YES	1		
		R1-5	003	1.06	15.0	YES	3		
Control	R2	R2-1	803	0.85	7.9	YES	1	8.68 ± 0.97	8.68 ± 0.97
		R2-2	565	0.87	8.1	YES	5		
		R2-3	053	0.82	7.0	YES	2		
		R2-4	609	0.94	12.5	YES	1		
		R2-5	299	0.87	7.9	YES	0		
Nucleotide	R3	R3-1	091	0.84	6.5	YES	2	7.02 ± 0.6	7.02 ± 0.6
		R3-2	081	0.79	5.2	YES	3		
		R3-3	086	0.85	7.7	YES	1		
		R3-4	894	0.87	8.8	YES	1		
		R3-5	046	0.81	6.9	YES	2		

2.3.1.2 Haddock broodstock

Two hundred and fifty 1.5-year-old fish (Ardtoe reared), which were first allocated to the same six tanks on November 18th 2001, formed the haddock broodstock (Table 2. 2). Water temperature, which was not chilled in this case, was also recorded daily. Tanks were assigned to either control or nucleotide diet, and feeds introduced on November 25th 2001. Haddock were fed these diets for two consecutive spawning seasons until July 2003.

Attempts were made to sex the haddock by using ultrasonography (Sonovet® 7.5 MHz transducer) but results proved inconclusive due to the limited development of gonads at this stage. It was assumed that with 50 and 26 fish randomly assigned to the 20 m^3 and 15 m^3 tanks respectively, there would be a suitable ratio of males to females in each tank. On 18th December 2001 a problem with water quality caused the loss of all the stock in one of the nucleotide tanks (R3) and 2 fish in tanks R1 and R2; reducing the number to 222. It was decided to empty F3 control tank and redistribute the fish remaining, 196, in the tanks (F1, R2 control and F2, R1 nucleotide) among all the tanks so as to maintain comparable stocks with regard to spawning and feed history. Therefore, between March 7th and 8th 2002, twelve fish from the remaining NT tanks were transferred to tank R3 and the same number moved from F1 and R2 to the F3, (the three control tanks). All the females were provided with Passive Integrated Transponder (PIT) tags at this stage (See Table 2. 2 to check fish distribution on March 2002 after mortalities). Investigations using ultrasound, as those by Martin-Robichaud & Rommens (2001), could have been conducted at this time but it is known that handling sexually mature gadoids prior to spawning can cause significant mortality (Cutts & Shields, 2001) so ultrasound was not carried out. It is well documented that fish are more susceptible to stress while in spawning condition, compared to other phases of their annual growth and reproductive cycle. Therefore, broodfish were left to spawn naturally and the ultrasound technique was used to determine the sex the fish at the end of the spawning season. Fish were sexed on 28th May 2002 but the sex of 16 fish remained uncertain out of the total 182.

Table 2. 2 Distribution of haddock broodstock between 18th November 2001 and 8th May 2003.

Diet	**Control**			**Nucleotide**		
Tank	F1	F3	R2	F2	R1	R3
18th November 2001						
Number of fish	50	26	50	50	50	26
Total biomass (g)	47195	41770	50860	46200	46530	39430
Mean weight (g)	943.9	1606.5	1017.2	924.0	930.6	1577.2
7th March 2002						
Number of fish	38	24	36	38	36	24
Total biomass (g)	46455	29490	44460	44479	41598	27912
Mean weight (g)	1222.5	1228.8	1235.0	1170.5	1155.5	1163.0
28th May 2002						
Number of fish	38	23	31	34	33	23
Total biomass (g)	36434	22198	33317	32605	30626	21220
Mean weight (g)	958.8	965.13	1074.7	956.0	928.1	922.61
18th February 2003						
Number of fish	8	8	8	8	8	0
Total biomass (g)	10120	10040	1118	9910	8510	0
Mean weight (g)	1265	1255	1397.5	1238.8	1063.8	0
8th May 2003						
Number of fish	7	7	8	8	7	0
Total biomass (g)	8070	8490	9605	9316	6524	0
Mean weight (g)	1152.9	1212.9	1200.6	1164.5	932.0	0

Further mortality events in two of the nucleotide tanks (12-20 September 2002, and 6-7 December 2002) and one of the control treatment tanks (15-20 December 2002), together with other losses associated with the stress of the first spawning season reduced the number of available broodstock. At the beginning of the second spawning season, on 18th February 2003, broodstock fish were redistributed within each dietary treatment to provide eight fish per tank (1:1 sex ratio) (Table 2. 2). Tank R3 was not restocked, due to insufficient females in the nucleotide treatment. Therefore, the trial carried on with three control tanks and two nucleotide tanks with four males and four females assigned to each of them. External colour-coded tags were put in the first dorsal fin of each female in order to individually monitor abdominal distension. This was hoped to identify females that had spawned during the previous night by observing any decrease in the abdominal distension of individual fish.

2.3.2 Gamete collection

Halibut and haddock are batch spawners; individual fish release multiple batches of pelagic eggs according to their ovulatory rhythm during the spawning season. However, egg collection protocols were different for both species. While halibut were manually stripped for eggs and milt, haddock were left to spawn naturally in the tanks and eggs were collected daily (Details in sections 2.3.2.1 and 2.3.2.2 respectively).

2.3.2.1 Atlantic Halibut

Halibut broodfish were manually stripped of eggs and milt. Stripping secures parental identity and good hygiene in contrast to natural spawning where parental identity can be difficult to establish and the risk of bacterial infections is higher since the eggs are shed directly into the water. The halibut is a multi-spawner, like most flatfish, therefore in order to maximise the yield of good quality eggs it was very important to closely monitor the ovulatory rhythms of individual females in order to obtain ripe eggs when stripped (Norberg *et al.*, 1991).

2.3.2.1.1 Monitoring ovulatory rhythms:

Although in previous studies (Norberg *et al.*, 1991) the ovulatory cycles have been shown to occur every 3 - 4 days, it depends on each individual. Therefore, it was important to determine it for each female so that effects from under- and over-ripening were minimised. Three 'tools' were used to determine the ovulatory rhythms:

- **Swelling abdomen**: females starting the spawning cycle were easily recognised by the increasingly swollen abdomen along the side of body. Therefore, the degree of abdomen distension was visually checked twice a day (morning and evening) throughout the spawning season.

- **Natural egg batch release**: in order to estimate the inter-ovulatory period and establish the stripping time as accurately as possible, females were allowed to release the first two batches of eggs (which are usually of poor quality) into the tank water. These were collected in egg-collectors at the tank outflow and provided an estimation of the ovulatory rhythm for subsequent batches of each individual female.

- **Egg chorion morphology**: Atlantic halibut egg chorion morphology varies depending upon the degree of ripeness. Thus in overripe eggs the chorion is dimpled and similar to 'golf-balls'; with 'scratches' if the egg is not ripe enough, and 'smooth' when the eggs have been collected at the right time. So the ripeness of egg batches can be determined by observing, under a binocular microscope, the chorion of halibut eggs collected in each stripping as in turbot (McEvoy, 1984).

2.3.2.1.2 Stripping procedure.

At the predicted time of ovulation the following procedure was followed. Two people were required to get into the tank to perform the stripping, a stripping table was lowered down into the tank and the female was gently guided onto it. Once the animal was in place one person handled it and applied a gentle pressure to the swollen abdomen, from back to front, while the other collected running eggs into a dry 2-L plastic jug (Figure 2. 2). After egg batch volume was assessed and the jug was put into a cold box to protect the eggs from excessive light and temperature.

The same procedure was repeated with two males from the same tank, though this time the gonopore was dried with a paper tissue before collecting the milt into 40-ml plastic beakers, in order to avoid activation of the milt by seawater. Neither male nor female fish were anaesthetised in any way during stripping and egg collection. Female and males were identified and date, time, tank temperature and egg volumes were recorded for each stripping. In case females released the batch of eggs into the tank water before being stripped an egg collector was

placed at the end of the outlet flow of each tank so that all the eggs were collected and egg production could be estimated accurately.

Figure 2. 2 Striping eggs from Atlantic halibut during the 2001 spawning season

2.3.2.1.3 Fertilisation:

Gametes, enclosed in the cold box, were taken into an egg incubation room at 5 °C and under a very dim light, in order to protect them from excessive light and temperature. A sample of eggs was taken for a volumetric estimation of the number of eggs per batch (§ 2.3.2.1.4.), while the rest of the eggs were carefully transferred to a 4-L plastic bowl. The standard procedure for wet fertilisation was then followed. Firstly milt from two stripped males was activated with UV sterilised, 5 μm filtered seawater and immediately pooled with the eggs into the bowl (the approximate ratio for 500 ml of eggs: 1:500 milt:water). The mixture was gently mixed and left for 20 minutes for egg hardening. After this time the eggs were transferred to a soft net and rinsed 4-5 times with 2 L of clean water. The eggs were finally transferred to a 70-L cylindro-conical polyethylene tank containing 70-L of clean, UV-sterilised, 5 μm filtered seawater at 5.5 – 6 °C and 35.5 ‰ salinity.

2.3.2.1.4 Egg production estimation

The number of eggs from each batch was also estimated as follows: the volume of each egg batch was recorded. From each egg batch, 2.5 ml samples were taken and the number of eggs was counted. This operation was repeated three times and the mean value recorded. Total number of eggs in each batch could therefore be estimated. Samples were also taken for dry weight, egg diameter and nucleotide content at this stage.

2.3.2.1.5 Sperm motility assessment

Milt from the Atlantic halibut is activated with seawater. Once activated, spermatozoids remain active for only 90 seconds. A drop of milt was placed on a microscope slide and a drop of water was added to the milt. With a second slide the mixture was spread into a thin layer. Milt was observed under a binocular microscope, and the percentage of motile sperm cells estimated. This procedure was repeated three times and the mean value recorded as sperm motility.

2.3.2.1.6 Egg drop-out assessment

Drop-out rate was expressed as the percentage of non-viable eggs from a batch. Drop-out rate was calculated 24 hours post-fertilisation, during the incubation period and total drop-out according to the following equation:

$$D = \frac{non - viable\ egg\ mass}{total\ egg\ mass} \times 100 \quad \text{[Eq.2. 1]}$$

2.3.2.2 Haddock

Egg protocols followed for haddock experiments were very similar to those previously used by Cutts and Shields (2001) for Atlantic cod.

2.3.2.2.1 Egg collection

Haddock females spawn several batches of small pelagic eggs during the spawning period. Haddock, particularly breeding females, are very sensitive to handling stress during the spawning season. Since haddock can spawn spontaneously in captivity, fish were left to spawn naturally and fertilised eggs were collected from the outflow of the holding tank with a plankton net. Discrete batches of eggs were collected at dusk by increasing the flow into the tank (e.g. 100 $L \cdot min^{-1}$ for a 150 m^3 tank) during the morning to flush out older eggs, then lowering the flow (to 70 – 80 $L \cdot min^{-1}$) for the rest of the day. The eggs were then collected with little contamination from older eggs.

Figure 2. 3 Haddock egg collection from plankton nets.

Since live eggs float, buoyant eggs were collected daily in 100-µm plankton nets at the end of the outflow pipes suspended in a 2-m^3 tank (Figure 2. 3). The plankton nets were lifted, concentrating the eggs in the 'cod end'. The 'cod end' was then untied, and the eggs were rinsed into a 25-L bucket for their transportation to the hatchery unit. This procedure was carried out for the six tanks every day through the spawning season.

Once in the hatchery unit, the water volume in the buckets was topped up to 20 L with seawater of at least 32 ‰ salinity and left for 10 minutes to allow all the viable eggs to raft on the surface. Buoyant eggs would float, while dead eggs sank. Various egg samples were then

immediately taken for dry weight (x 3), for nucleotide analysis (x 3) -which were stored in liquid nitrogen- as well as for determination of fertilisation rates and developmental stage. Images of eggs were also captured for morphometric calculations each day from a control and a nucleotide tank (§ 3.3.2.3).

2.3.2.2.2 Staging of eggs

A subsample of each collected egg batch was taken and developmental stages were then examined according to criteria for cod as described by (Russell, 1976; Thompson & Riley, 1981; Makhotin *et al.*, 1984). Mixed batches, containing eggs at different developmental stages were not incubated. Otherwise, a range of egg ages would cause problems during egg incubation since old eggs would hatch before younger ones.

2.3.2.2.3 Egg production estimation

Egg production of all the batches, mixed and homogeneous, was estimated. Viable eggs were removed from the water surface of the 25-L buckets and transferred into a fine mesh hand net immersed in water. The net was then dipped into a 3-L plastic bucket and eggs were disinfected with a 4 ‰ solution of Kickstart® for 60 seconds. The eggs were rinsed in clean UV-sterilised and 5 µm-filtered seawater, decanted into a tared 5-L acrylic container filled with 3 L of clean UV-sterilised and 5-µm filtered seawater, and finally weighed. Dead eggs were also weighed.

Previous work (Gara & Shields, 1997) had estimated that there are approximately 500 eggs in one gram of eggs. Egg counts were also carried out and fifty eggs were randomly sampled and dried with tissue paper, weighed and the number of eggs per gram of sample estimated. This operation was repeated three times and the mean value recorded. The total number of viable, fertilised eggs could then be calculated.

2.3.2.2.4 Egg dropout assessment

As mentioned above, dead eggs, which sank, were also weighed on a tared scale and weights recorded. Dropout rate was expressed as the percentage of non-viable eggs from a batch according to equation 2.1.

2.3.3 Broodstock Feeding

2.3.3.1 Halibut diets and feeding regime

A nucleotide-enriched and a control diet were tested in the halibut study. A diet mix composed of Fish Meal –Norse LT94 (5 kg), Shrimp meal (500 g), vitamins (50 g), betaine (100 g), mackerel oil (500 g), minced squid mantle (2 kg) and seawater (5 L) was prepared and divided evenly by weight. The nucleotide additive (Optimun, Chemoforma Ltd, Switzerland), which contains yeast extracted nucleotides (AMP, GMP, UMP, CMP and TMP), nucleotide precursors and RNA, was added to 50 % of the diet at a concentration of 0.5 % of wet weight, prior to the administration of the diet mix into a sausage extrusion process. The combined inclusion level of nucleotides in the nucleotide diet was then 0.075 %. Equipment was cleaned to avoid contamination between the nucleotide and the control diets during mixing and manufacture of the sausages.

Fish were fed to satiation on these diets every three days from 21st October 2000 until July 2001. Feeding response was monitored at each feed and consumption recorded for each tank. Fish were weighed at the start of the trial, and at the end of each individual's spawning season. Feed consumption per month was calculated according to the following equation:

Food ration = [(kg feed/ kg fish in tank) x 100]/number of feeds per month [Eq.2. 2].

2.3.3.2 Haddock diets and feeding regime

Haddock were fed to satiation every other day from 25th November 2001 until July 2003, for two consecutive spawning seasons. Diets were provided by Ewos Innovation (Norway), details of them are given in Table 2. 3; both were isocaloric, only differing in the addition of the nucleotide additive (Optimun, Chemoforma Ltd, Switzerland) to one of them. Feeding response was also monitored at each feed and consumption recorded in each tank. The combined inclusion level of nucleotides in the nucleotide diet was then 0.12 %. During the second spawning season, as there were only eight fish per tank an attempt was made to record individual feed consumption but finally it was not possible. Fish were weighed at the beginning and end of each spawning season.

Table 2. 3 Composition of the control and the nucleotide-enriched diets used during the haddock trial

	Control	Nucleotide
Protein (%)	50.90	50.80
Lipids (%)	11.70	12.30
Ash (%)	7.80	7.80
Water (%)	12.20	11.70
NFE[1] (%)	16.90	16.10
Vitamins & Minerals (%)	0.5	0.5
Nucleotide additive(%)	0	0.8
Gross E (KJ/g)	19.6	19.6

[1]: Nitrogen-free extracts

2.3.4 Broodstock condition

2.3.4.1 Halibut

In order to calculate condition factor, all broodfish females were measured for length and weight. Halibut females were placed on a framed net, suspended from a scale and fork length (from mouth to caudal fin) and total weight was recorded. Thereafter, Fulton's condition factor was calculated according to the following equation:

$$K = \frac{W}{L^3} \times 100 \qquad \text{[Eq.2. 3]}$$

where **K** is the condition factor, **W**: mass (g), and **L** is length (cm). The heavier a fish for a given length, the higher its condition factor (K). Gonadosomatic and hepatosomatic indices could not be studied since broodfish were not culled at the end of the experiment due to their high value.

2.3.4.2 Haddock

Broodfish were measured for length and weight at the beginning and end of the spawning season and the Fulton's condition factor calculated according to the equation 2.3 (§ 2.3.4.1). At the end of the experiment, when the second spawning season was finished, all the remaining females were culled and gonadosomatic and hepatosomatic indices calculated according to the following equations.

$$G.S.I. = \frac{gonad \quad weight}{body \quad weight} \times 100 \qquad \text{[Eq.2. 4]}$$

$$H.S.I. = \frac{liver \quad weight}{body \quad weight} \times 100 \qquad \text{[Eq.2. 5]}$$

where **GSI** and **HSI** are gonadosomatic and hepatosomatic indices respectively, and gonad, liver and body weights are expressed in grams (g).

2.3.5 Atresia studies

Since atresia is a degenerative process occurring in the ovary, it affects broodstock fecundity. Therefore, ovarian samples from the remaining haddock fed both dietary treatments were studied using light microscopy in order to check for levels of atresia at the end of the second spawning season.

2.3.5.1 Light microscopy.

Ovarian samples were collected from all the surviving haddock at the end of the second spawning season (May 2003). Cuboidal sections of the ovary were cut with a scalpel, and smaller sections of these (approx. 1 cm^3) fixed and processed for histological analysis.

a) Fixation:

Sections of ovary were stored in at least 10 times their volume of buffered formalin for 24 h. After this time, the buffered formalin was renewed, and the sample stored in a cool place until processing. Buffered formalin was prepared as follows:

$Na\ H_2PO_4$ ..... 3.5 g

Na_2PO_4 ..... 6.5 g

Formalin (40% formaldehyde)..... 100 ml

Deionised water ..... 900 ml

b) Dehydration infiltration embedding and mounting:

Inside the fume cupboard, small pieces of tissue (< 1 cm x 1 cm) from the different samples were dissected out, placed in separate containers and dehydrated in a series of ethanol solutions of increasing strength according to the following protocol:

30% alcohol ..... 1 h

50% alcohol ..... 1 h

70% alcohol ..... 1 h

90% alcohol ..... 1.5 h

95% alcohol ..... 1.5 h

100% alcohol (bath 1)..... 1.5 h

100% alcohol (bath 2)..... 1.5 h

100% chloroform (bath 1)..... 1.5 h

100% chloroform (bath 2)..... 2 h

Wax bath 1 ..... 2 h

Wax bath 2 ..... 3 h

Wax bath 3 ..... 3 h

After infiltration, samples were placed individually in labelled moulds and one by one embedded with fresh wax (Agar Scientific®). Wax was left to solidify and cool down on a cold plate for 2 hours.

c) Sectioning:

Once the wax solidified, samples were sectioned at a thickness of 8 μm with a microtome (Jung, AG Heidelberg) at an angle of incidence of 12 °. Selected sections were floated in a water bath at 53 °C and extended on Polysine™ microscope slides (BDH Laboratory Supplies, U.K.) using glycerine albumen as an adherent. Samples were dried out on a hot plate at 40 °C for one day before staining.

d) Staining:

Sections of ovarian tissue mounted on microscope slides were stained with haematoxylin and eosin with the following procedure:

Xylene 100%..... 5 min

Xylene/ Ethanol 50:50 ..... 5 min

Ethanol 100%..... 2 min

Ethanol 95%..... 2 min

Ethanol 90%..... 2 min

Ethanol 70%..... 2 min

Ethanol 50%..... 2 min

Haematoxylin..... 5 min

Wash in tap water..... 5 min

Acid alcohol 70%..... 7-8 sec

Potassium acetate..... 5 min

Wash in tap water..... 5 min

Eosin ..... 7 min

Absolute alcohol I..... 2 sec

Absolute alcohol II..... 2 sec

Xylene..... 5 min

Mounting xylene..... up to 1 h

The slides were kept in xylene until a cover slip was mounted using DPX resin.

e) Image acquisition:

Samples were observed through a Carl Zeiss Axioskop2-plus binocular microscope connected to a digital camera (Carl Zeiss Axiocam HRc) and to a computer. An x0.63 lens was fitted in the neck piece connecting the camera to the microscope and the Carl Zeiss Axiovision 3.1 image analysis software was used to acquire all images.

2.3.5.2 Stereological analysis

This method was first applied to fish to estimate fecundity in herring, Dover sole and mackerel (Emerson *et al.*, 1990). It is based on the Delesse principle (Delesse (1847), cited in Emerson *et al.* 1990), which states that the fractional volume (V_i) of a component (i) is proportional to its fractional cross sectional area (A_i). In this thesis, the method was applied to compare the volume fraction of atretic oocytes (V_i) in ovaries of haddock at the end of the second spawning season.

The methodology employed was a modification of that of Emerson *et al.* (1990) and the protocol used later on with tilapia (Coward & Bromage, 2002). Validation of the technique can be found in the same sources. The proportional area (A_i), and hence the proportional volume (V_i), occupied by atretic oocytes was determined by a point counting technique (p_i), using a Weibel grid (which overlays the oocytes). The total number of points overlaying a particular oocyte class, atretic in this case, was divided by the number of points on the grid (p_t).

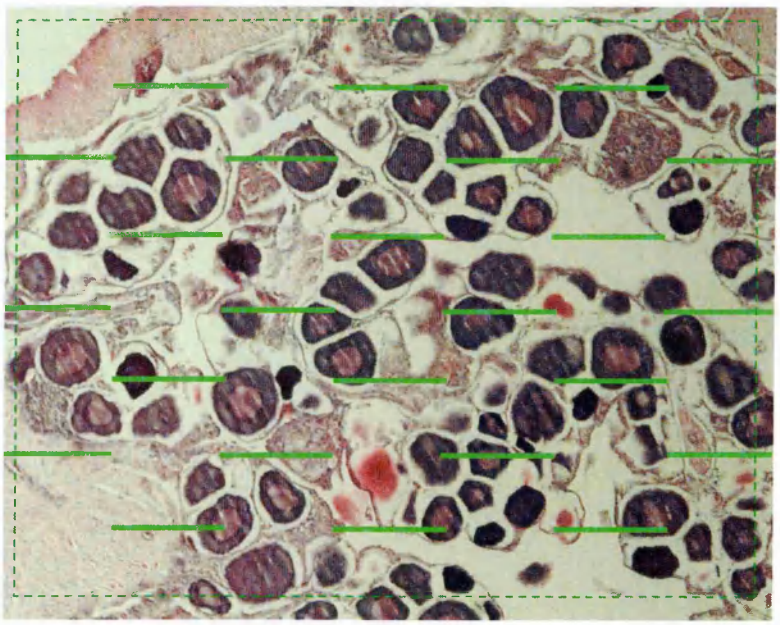


Figure 2. 4 Example of a Weibel grid overlaid on the image of ovarian section showing point counts to determine the partial volume (V_i) of oocytes in the ovary of haddock. In this grid there are 24 bars and the end of each bar inside the frame represents a test point (n = 42)

Ovarian samples, prepared as described in section 2.2.5.1, were observed through the binocular microscope described above and images recorded. Series of five slides containing five sections from the same tissue sample where studied. A protocol was established by which two fields were recorded. Thereafter, a Weibel graticule with 42 points was projected on top of each photograph for image analysis (see Figure 2. 4). It is accepted that a total of 300 grid points, spread over a number of sections and fields, is required to adequately establish a variance of about 5% (Weibel (1979) cited in Coward & Bromage 2002).

As cited in Coward and Bromage (2002) "*mathematics demonstrates that the area occupied by a particle of interest is equal to its volume assuming a section of zero thickness*". Therefore, the

area fraction (Ai), and hence the volume fraction (Vi) of atretic oocytes was calculated according to the formula:

$$Vi=Ai= pi / pt$$

The number of points of the grid overlaying atretic oocytes was recorded and the volume fraction calculated for each image. The average of all the volume fractions of atretic oocyte measured per fish was recorded as the percentage of atretic oocytes in the ovary of each female.

The incidence of atresia was calculated as the number of fish with atresia over the total number of fish at the end of the second spawning season for each dietary treatment and data expressed as a percentage.

2.3.6 Data analysis

Statistical analysis of the data was carried out using Minitab Statistical Software Release 14.0 and SAS Release 8.02. Parameters expressed as percentages or proportions tend to form a binomial, rather than a normal, distribution; therefore variables such as floating fraction of eggs, egg dropout and atresia levels were first arcsin-transformed (Zar, 1984) and thereafter the assumptions for the analysis of variance (ANOVA) and *t*-test were checked. Normality and homoscedasticity were studied for all the parameters. If the variable was normally distributed and homogeneity of variances criteria were fulfilled then an ANOVA test was carried out in order to study possible differences in spawning performance between broodstock fed the control diet and the NT-enriched diet. A multiple comparison Tukey test followed the ANOVA test in order to study differences between tanks. When variables were not normally distributed, a Box-Cox transformation was applied to the data in order to fulfil the ANOVA assumptions (Sokal & Rohlf, 1981). When these assumptions were not met, non-parametric methods such as Mann-Whitney test and Mood's median test were also used if non- normal parameters had equal and unequal variances respectively.

Correlation and regression analysis (linear and non-linear) were used to study the relationship between variables. The relations between the different variables and seasonal parameters such as date, tank temperature and batch number were compared first. Since regression and correlational methods serve as the basis for General linear models (GLM), the seasonal variable that correlated best with the parameter studied (i.e. with the highest correlation value "r") was used as a covariate for GLM and analysis of covariance (ANCOVA). GLM analysis was used in order to take into account all the parameters that could influence the response of the variable. The main difference in the statistic analysis of the data from the halibut and haddock trials lay in the GLM analysis and was mainly a consequence of the experimental design. As explained in section 2.3.2, eggs were manually stripped from each spawning halibut female, whereas haddock eggs were collected from the plankton nets daily. Therefore it was not possible to determine the parental origin of the eggs. Consequently, basically two GLMs were used to study the different parameters:

a) In the halibut trial: $y = f$ (Diet Tank[Diet] Female Date); where Diet, Tank and Female are class variables and Date is a covariate. This would indicate that the dependent variable "y" (e.g. egg production) could be a function of the diet, tank, female and the date. Tank effects within treatments were studied by studying tank nested with diet (Tank [Diet]).
b) In the haddock trial the term "female" was unknown so it had to be removed from the model: $y = f$ (Diet Tank[Diet] BN); where Diet and Tank are class variables and Batch Number (BN) is a covariate and analogous to date.

When comparing both models, it is easy to realise that two different covariates were used depending on the trial: date and batch number in the halibut and haddock trials respectively. Since some egg batches of halibut were missed and dropped in the tank before the stripping was carried out, date was used as a covariate instead of batch number. In the haddock trial batch

number was selected since all the egg batches were collected daily and batch number correlated better than date with all the parameters studied.

Significance level was $p < 0.05$. All the results are given as means ± standard error of the mean (SEM).

2.4 Results

2.4.1 Feed consumption

2.4.1.1 Atlantic halibut

Feeding response was monitored at each feed and consumption recorded in each tank. Feed intake was calculated according to Eq. 2.2 and followed a normal pattern for Atlantic halibut broodstock (Figure 2. 5), with food ratios declining at the end of the main vitellogenic period (November), to very low values during pre-spawning and spawning (January to April). Feeding response increased at the end of the spawning season. There was no significant difference in feed intake between the two treatments ($p = 0.521$).

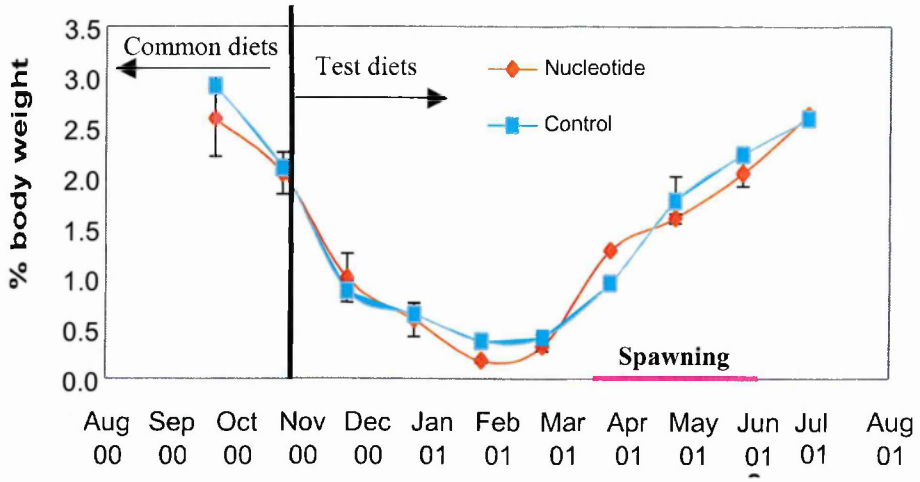


Figure 2. 5 Halibut food consumption (expressed as % body weight per feed on a wet weight basis) of the control (blue) and the nucleotide (red) diets. Vertical bar indicates beginning of the trial (21st October 2000). (Mean ± SEM).

2.4.1.2 Haddock

Food ratios, calculated using equation 2.2, declined before the beginning of the spawning season in both years, and fish hardly ate during this time (Figure 2. 6). Feeding response reached a normal level at the end of the spawning seasons. There was no significant difference ($p = 0.363$) in the feed intake between both treatments over the two spawning seasons. The big difference in October-November 2002 between both dietary treatments was due to large differences in the feed intake between the two remaining nucleotide tanks, one of them being overfed.

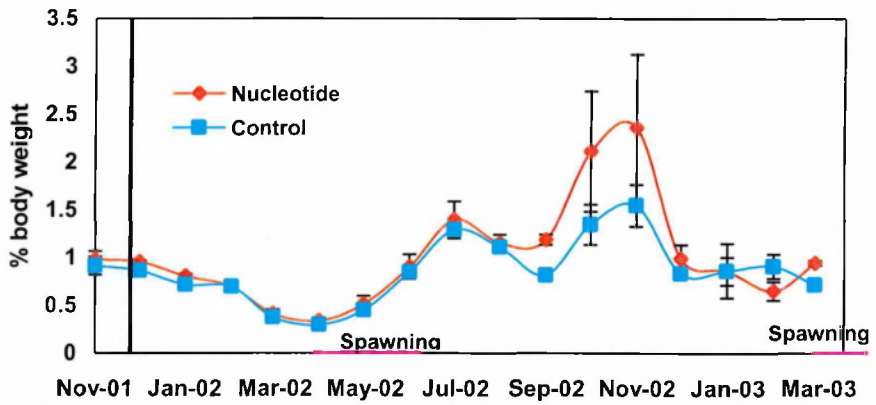


Figure 2. 6 Haddock feed offered expressed as % of body weight over the 2002-03 spawning seasons. Vertical line indicates beginning of the trial (25th November 2001). (Mean ± SEM).

2.4.2 Spawning performance

2.4.2.1 Atlantic halibut

Halibut broodstock began spawning in the middle of March 2001 (14th/03/2001) and continued until the beginning of June 2001 (3rd/06/2001). Egg production started first in one of the tanks fed the NT diet (F2), although in the rest of the tanks the fish started spawning approximately on the same date, apart from a control tank that was delayed in comparison to the others (Figure 2. 7, Figure 2. 9). The total spawning period and number of strippings per female had slightly higher values for the NT versus the control treatment (2.9 ± 0.60 vs. 2.6 ± 0.40 respectively)

although not significant, while the individual spawning periods were similar for both treatments (C: 27.23 ± 3.67 vs. NT: 27.60 ± 4.07) (Table 2. 4). Egg production in each tank followed a bell-shaped distribution, peaking around mid April, excepting nucleotide tank F2 that started earlier and had its highest values by the end of March (Figure 2. 7).

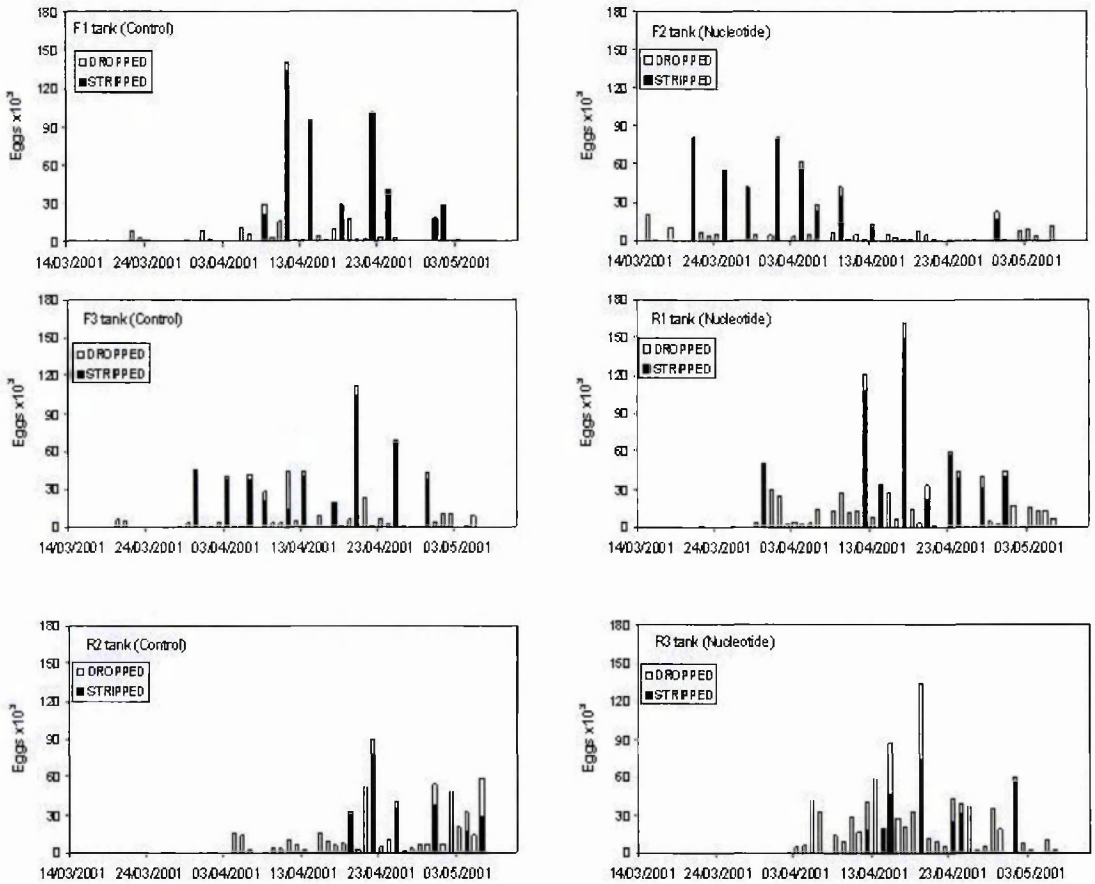


Figure 2. 7 Daily egg production in the different Atlantic halibut broodstock tanks (control on the left and nucleotide on the right). The data shown in the figure represent all the spawns and all the eggs dropped in the tank and obtained from the egg collectors at the outflows.

Table 2. 4 Spawning performance of female Atlantic halibut broodstock in the two dietary treatments. Mean ± SEM. Significant difference is denoted by *.

Treatment	Total spawning period (days)	Individual spawning period (days)	No. of strippings	No. strippings per female	Rel. batch fecundity per female (No. eggs/ kg/ batch)	Relative fecundity (Total No. eggs/ kg spawning females) *
Control	73	27.23 ± 3.67	35	2.6 ± 0.40	4,436 ± 312	18,294
Nucleotide	78	27.60 ± 4.07	31	2.9 ± 0.60	4,918 ± 528	23,232

Overall, 27 out of 30 halibut females produced eggs, 14 out of 15 on the NT diet and 13 out of 15 in the control group. During the spawning season, 66 manual strippings were carried out; 35 from females on the nucleotide diet and 31 from the controls. Despite the fact that almost all the female fish spawned, not all of them could be stripped for eggs (Table 2. 1). Thus, the gonopore became blocked after releasing the first two egg batches in females coded F2-2 and R2-5 and ovulatory rhythm could not be determined; female R1-2 did not show any evidence of abdomen distension, but released eggs once placed on the framed net (ovulatory rhythm could therefore not be precisely determined). Total egg production from all the tanks, including eggs from stripping and the egg collectors) at the end of the spawning season was 4.6 million eggs. Egg yield was 30% higher in the nucleotide tanks compared to the controls (i.e. NT: 2.6 million vs control: 2 million). In order to eliminate any possible bias derived from the size of the fish in the different tanks, relative fecundity was calculated. Egg production was standardised according to the spawning biomass in each tank (non spawning biomass excluded) and expressed as number of eggs per kilogram of spawning female in the tank. Relative fecundity at the end of the spawning season was significantly higher ($F = 22.22$; $p = 0.009$) in the NT tanks, producing 23,232 eggs per female kg compared to 18,294 eggs per female kg in the control ones (Table 2. 4 and Figure 2. 9). No significant difference was found in the batch relative fecundity between both diets (C = 4,436 eggs/kg/batch, NT = 4,918 eggs/kg/batch; $F = 0.00$, $p = 0.998$). Data were then pooled and a linear regression analysis revealed that this parameter significantly decreased along the spawning season ($r = -0.121$, $p=0.020$) (Figure 2. 8).

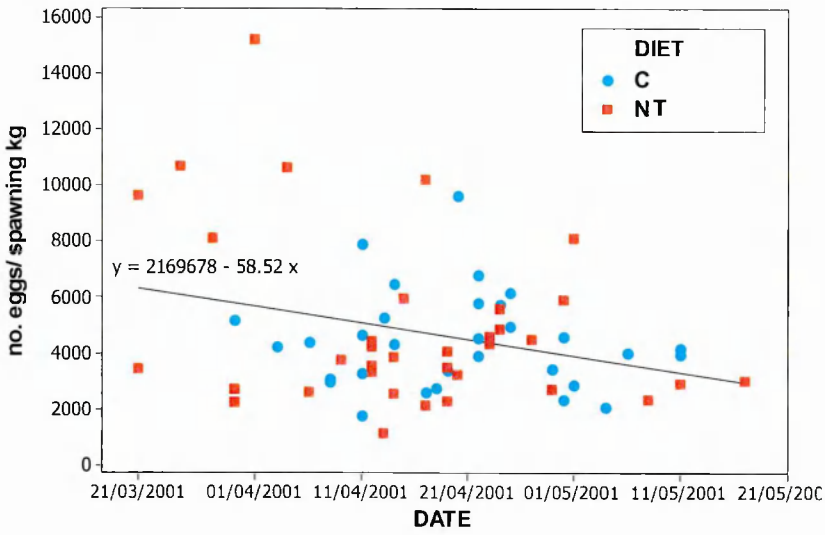


Figure 2. 8 Batch relative fecundity of Atlantic halibut along 2001 spawning season. Control (blue) and nucleotide (red) diets.

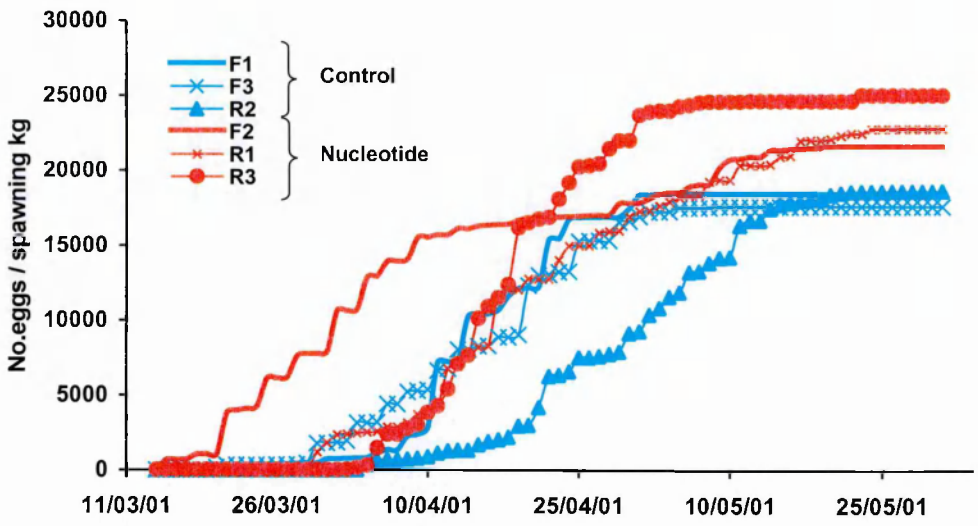


Figure 2. 9 Cumulative relative fecundity in the different tanks containing Atlantic halibut on control (blue) and nucleotide (red) diets.

The potential effects of the NT-enriched diet on milt motility were studied. In a scale from 0 to 10 (lowest and highest motilities respectively), there was no significant difference between both diets (C = 6.67 ± 0.32, NT = 6.44 ± 0.40; p = 0.65). As expected, milt motility decreased along the spawning season (r = - 0.543, p = 0.000) and this effect was observed in all the tanks (Figure 2. 10). There was a tank effect within diet, and the milt motility decreased more slowly in tanks F2 (NT) and F3 (Control) when compared to the other groups (Figure 2. 10). This difference in

milt quality, in terms of motility, was due to the fact that spawning activity in these two tanks occurred earlier when compared to the other tanks.

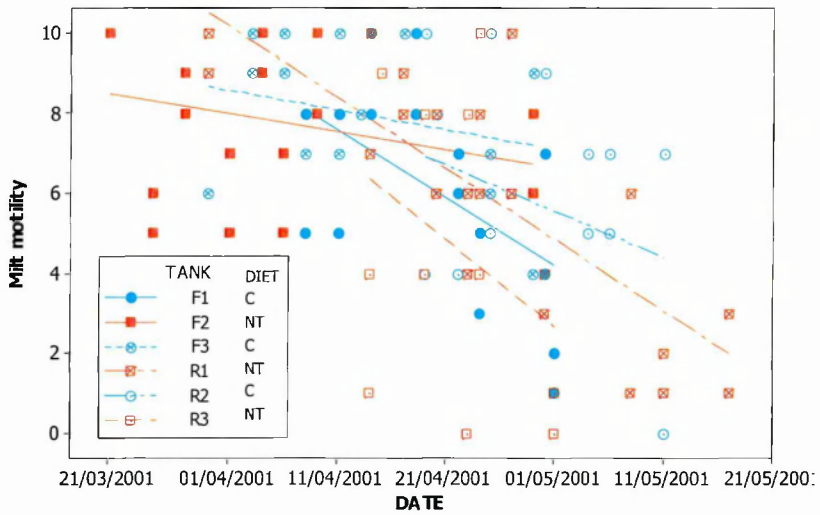


Figure 2. 10 Milt motility of Atlantic halibut in the different tanks along the spawning season. Control tanks in blue and nucleotide ones in red.

As mentioned above, drop-out 24 hours post fertilisation, during the incubation period and total drop-out were calculated. Results are summarised in Table 2. 5 and correlations with all the parameters affecting drop-out in Table 2. 6.

Table 2. 5 Drop-out rates of Atlantic halibut egg batches. Diets did not differ significantly. Mean ± SEM.

Parameters	Control	Nucleotide	P – value
Drop-out 24h (%)	42.77 ± 6.22	31.81 ± 4.57	0.196
Incubation Drop-out (%)	41.30 ± 2.39	37.01 ± 5.35	0.470
Total drop-out (%)	65.78 ± 5.07	58.53 ± 5.77	0.386

Drop-out 24h post fertilisation (D24) was not significantly different between diets ($F = 2.08$, $p = 0.1963$), and no correlation could be found with date. However, D24 was significantly negatively correlated with egg density ($r = -0.3488$, $p = 0.0203$) and fertilisation rate ($r = -0.5574$, $p < 0.0001$). In order to confirm this, a stepwise regression was carried out using milt motility, batch size, fish condition, weight and length, egg dry and wet weight, egg volume, egg density (EggD) and fertilisation rate (FR) (Chapter 3 for details) as potential predictors of D24. The regression equation that fitted the data best (i.e. with the highest R^2 – adjusted) was:

$$D24 = 1 - 0.55\ FR - 0.303\ EggD, \qquad p = 0.000;\ R^2\ (adj) = 0.3432$$

The main parameters that influenced D24 were fertilisation rate and egg density. A combination of high FR and eggs with specific gravities between 1.05 – 1.1 $g \cdot ml^{-1}$ led to the lowest dropout rates after fertilisation (Figure 2. 11).

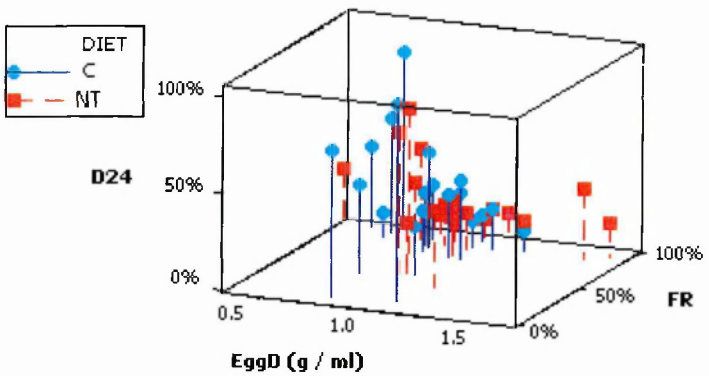


Figure 2. 11 Dropout 24 hours post fertilisation (D24) versus fertilisation rate (FR) and egg density (EggD) in Atlantic halibut eggs from control (C) and nucleotide (NT) diets. No significant difference between diets.

No significant difference in the incubation dropout (DI) was found between diets ($F = 0.45$, $p = 0.470$). DI was correlated with batch volume ($r = 0.4079$, $p = 0.0386$), egg density ($r = -0.4139$, $p = 0.0356$) and also slightly with female condition ($r = -0.103$, $p = 0.0077$). Total dropout (TD) was also not significantly different between the control and nucleotide diets ($F = 0.78$, $p = 0.386$) and when data were pooled it was correlated with batch volume ($r = 0.5744$, $p = 0.0021$), fish size ($r = 0.4725$, $p = 0.0148$) and again with egg density ($r = -0.6317$, $p = 0.0005$).

Table 2. 6 Pearson correlation values (r) of all the parameters influencing dropout in Atlantic halibut eggs. Dropout 24 hours after fertilisation (D24); incubation dropout (DI) and total dropout (TD); ns: no significant correlation.

	D24	DI	TD	Batch volume	Fish size	Female condition	EggD
D24	1						
DI	ns	1					
TD	0.643	0.6737	1				
Batch volume	ns	0.408	0.574	1			
Fish size	ns	ns	0.472	0.458	1		
Female condition	ns	- 0.510	ns	ns	- 0.400	1	
Egg density	- 0.3488	- 0.4139	- 0.6317	ns	ns	ns	1

2.4.2.2 Haddock

Haddock started spawning on 25th March and 6th March in 2002 and 2003 respectively (i.e. first and second spawning seasons) and continued until 12th June 2002 and 6th May 2003. Egg production started in all the tanks approximately at the same time in each season. During the first season, 359 egg batches were collected, 185 from the control tanks and 174 from the nucleotide tanks, with a total egg production of 41.4 million eggs. The number of egg batches collected in 2003 was 189 -125 from the three control tanks and 64 from the remaining two nucleotide tanks. Total egg production was 10.26 million eggs (7.69 million from the three control tanks and 2.57 million from the two NT tanks).

All the parameters used to compare the spawning performance between the control and nucleotide diets are summarised in Table 2. 7. The total spawning period was slightly longer (6 days) for the control group in 2002 (79 vs 73 days) while the duration was the same for both diets in 2003. No significant differences were found in the number of batches collected per female between the two diets in either year ($p > 0.05$). Egg production was significantly higher in the control tank in 2002 (8.12 ± 1.17 million vs 5.71 ± 0.19) and in 2003 (2.56 ± 0.11 vs 1.28 ± 0.11) as a result of a higher number of females spawning in the control tanks. Furthermore, the mortality events during 2002 and 2003 (see Appendix II) reduced the broodfish stocks and consequently, the number of eggs produced was lower in 2003 when compared to 2002.

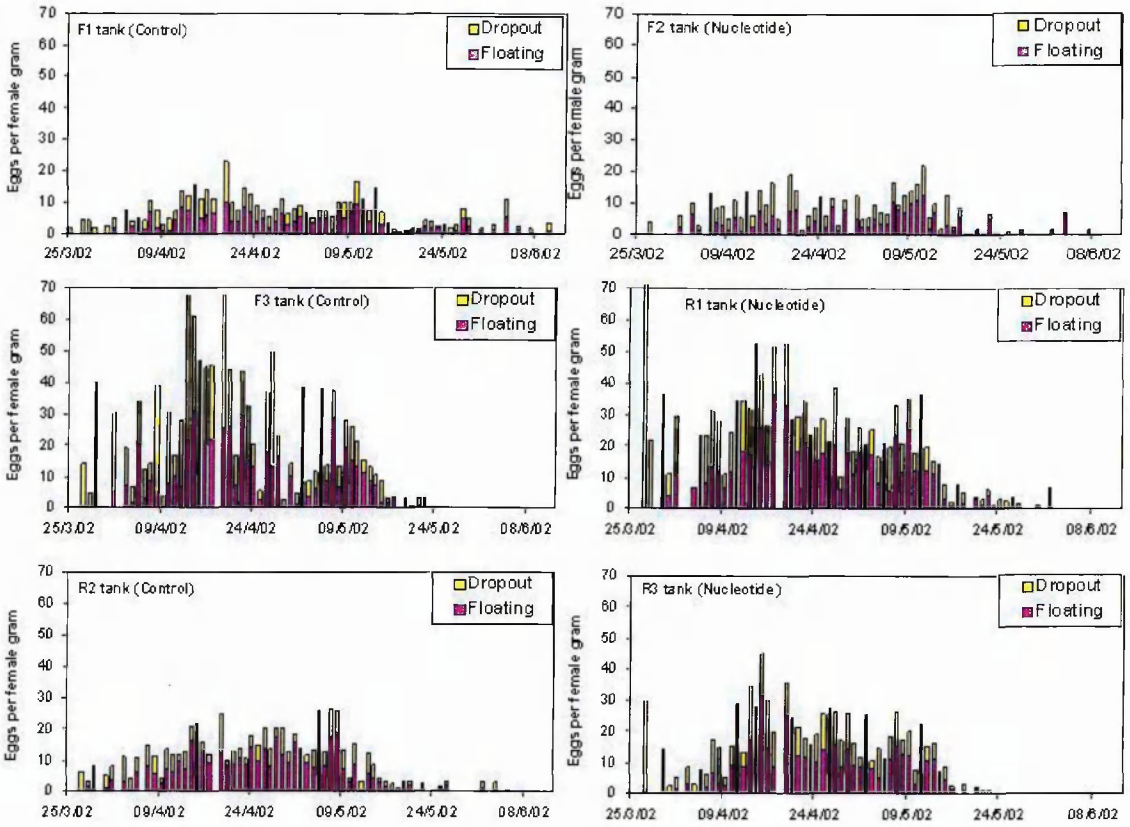


Figure 2. 12 Daily egg production in the different haddock broodstock tanks in 2002. Control on the left and nucleotides on the right.

Batch relative fecundity varied greatly, ranging from 510 to 88,580 eggs/kg in 2002 (Figure 2. 12) and 500 to 140,200 eggs/kg in 2003 (Figure 2. 13). Variation in batch relative fecundity over the season was slightly different in 2002 and 2003. Thus, in 2002 the highest values occurred mainly in the egg batches from the middle of the season, while batch relative fecundity decreased over the spawning season in 2003 (Figure 2. 14). Differences in total relative fecundity of control and NT diets were not significantly different in 2002 (C: 853.44 ± 195.55 vs NT: 983.73 ± 271.79) nor in 2003 (C: 563.62 ± 41.62 vs NT: 493.93 ± 102.89) (Figure 2. 15), despite being over 13% higher in the NT diet in 2002. However, batch relative fecundity was higher for the NT diet in 2002, with differences being significant ($W = 30959.0$; $p = 0.0172$) (Figure 2. 16).

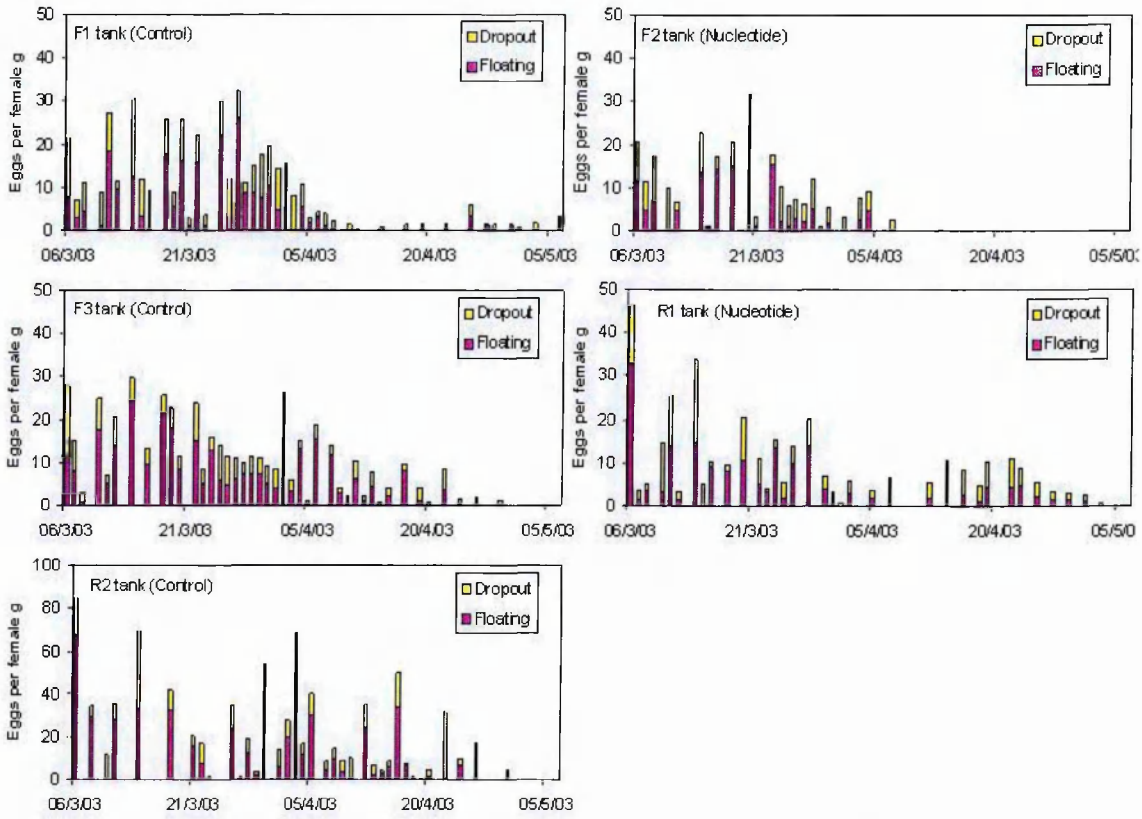


Figure 2. 13 Daily egg production in the different haddock broodstock tanks in 2003. Control on the left and nucleotides on the right. Note scale in tank R2 is different.

Table 2. 7 Haddock spawning performance parameters of the different diets along 2002 and 2003 seasons. Significant differences are denoted by *. (Mean ± SEM).

	2002		2003	
Parameters	Control	Nucleotide	Control	Nucleotide
Starting date	25 March	26 March	6 March	6 March
Finishing date	12 June	7 June	6 June	6 June
Total spawning period [1]	79	73	61	61
Mean spawning period [1]	69.33 ± 6.49	66 ± 4.58	59 ± 2	50 ± 11.
No. batches collected	61.67 ± 4.10	58 ± 2.08	41.67 ± 2.33	32.00 ± 5.00
No batches per female	6.73 ± 2.04	9.29 ± 3.09	10.64 ± 0.72	12.17 ± 3.17
Total egg yield per tank[2]	8.12 ± 1.17 *	5.71 ± 0.19	2.56 ± 0.11 *	1.28 ± 0.11
Total relative fecundity [3]	853.44 ± 195.55	983.73 ± 271.79	563.62 ± 41.62	493.93 ± 102.89
Batch relative fecundity [3]	12.36 ± 0.93	15.22 ± 1 *	13.34 ± 1.36	14.98 ± 2.09
Floating eggs per batch (%)	47.60 ± 1.68	46.79 ± 1.66	50.22 ± 2.39 *	41.64 ± 3.26
Dropout per tank (%)	52.39 ± 2.61	53.41 ±1.17	38.28 ± 4.04	49.65 ± 4.60

[1]: Days; [2]: million eggs; [3]: eggs per female gram

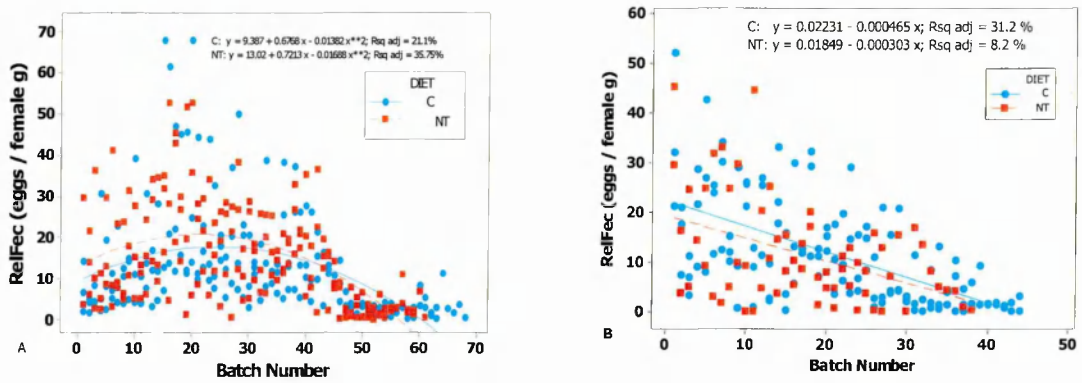


Figure 2. 14 Seasonal variation of batch relative fecundity in haddock along 2002 (A) and 2003 (B).

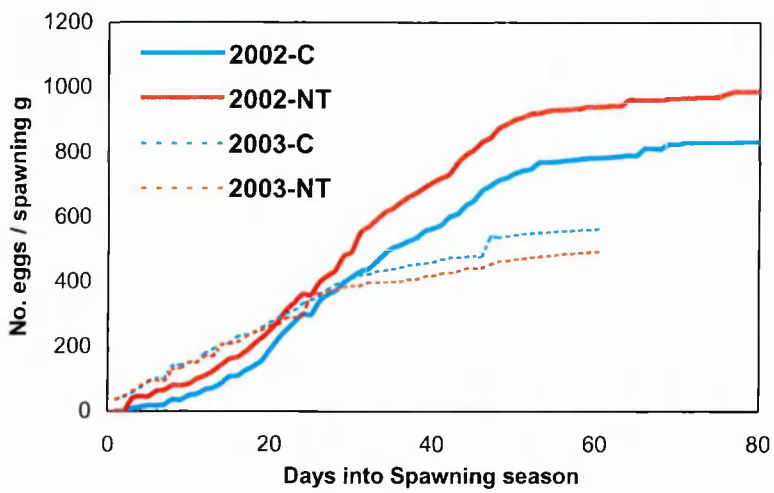


Figure 2. 15 Total relative fecundity (number of eggs per gram of spawning female per tank) with control (blue) and nucleotide (red) diets along 2002 (continuous line) and 2003 (discontinuous line).

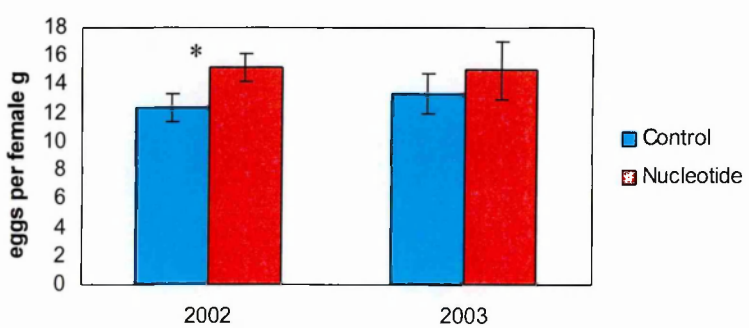


Figure 2. 16 Batch relative fecundity (number of eggs per gram of spawning female) in 2002 and 2003 for control (blue) and nucleotide (red) diets. * denotes significant differences between diets. (Mean ± SEM)

Floating fractions were similar for both diets in the first year (C: 47.60 ± 1.68, NT: 46.79 ± 1.66; F = 0.11, p = 0.677), however were significantly different in 2003 (C: 50.22 ± 2.39 vs NT: 41.64 ± 3.26; F = 4.43, p = 0.026) in favour of the control diet (Table 2. 7). No statistical difference was found in total batch dropout (F = 3.33, p = 0.165). Variation along the season was slightly different between 2002 and 2003. The floating fraction in 2002 followed a dome-shape distribution (C: F = 48.54, p = 0.000; NT: F = 25.22, p = 0.000) while in 2003 it decreased over the spawning season (C: F = 9.98, p = 0.002; NT: F = 5.25, p = 0.025) (Figure 2. 17).

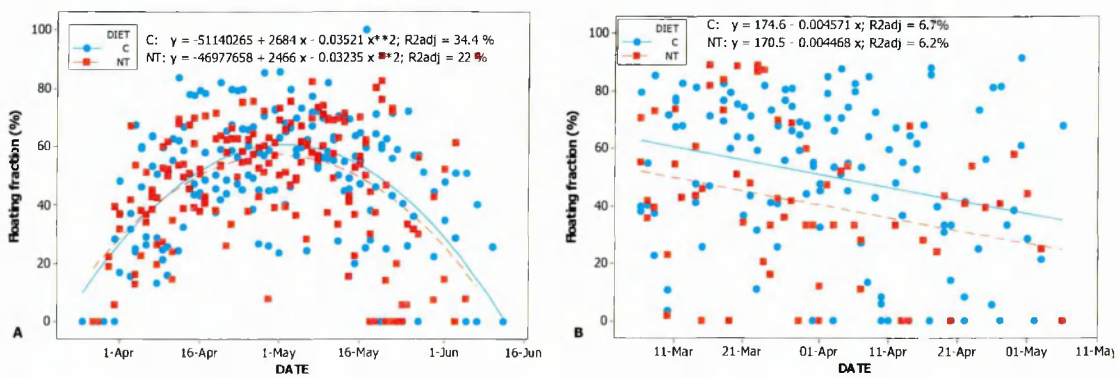


Figure 2. 17 Seasonal variation of the floating fraction of haddock eggs over 2002 (A) and 2003 (B).

2.4.3 Broodstock condition

2.4.3.1 Atlantic halibut

As explained in section 2.3.4.1 Fulton's condition factor was used to evaluate the condition of all the females at the end of the spawning season. Despite the higher fecundity of the females on the nucleotide diet, there was no significant difference (F = 0.151; p = 0.701) in the condition factors at the end of spawning between both diets (Control: K=1.33 ± 0.04 vs. nucleotide: K=1.30 ± 0.06 ($g \cdot cm^{-3}$)).

2.4.3.2 Haddock

The condition of the remaining haddock broodfish was assessed at the end of each spawning season. However, no significant differences were found between dietary treatments in 2002 ($F = 0.14$; $p = 0.710$) or in 2003 ($W = 293.0$, $p = 0.999$) (Table 2. 8). Since the exact number of males and females at the beginning of the first spawning season (2002) was unknown, weight loss could not be calculated separately for each sex as in 2003. Weight loss at the end of the first and second spawning season did not differ between diets (2002: $F = 0.01$, $p = 0.944$; 2003: females: $F = 0.33$, $p= 0.607$; males: $W = 8.0$, $p = 0.773$; Table 2. 8).

As mentioned above, at the end of the second spawning season all the females were culled and the gonadosomatic and hepatosomatic indices (GSI and HSI respectively) calculated according to the equation described in section (2.3.4.2). Initially no significant differences were found in the GSI and HSI between fish fed the control and the nucleotide diets (GSI: 1.6840 ± 0.497 % vs 2.57 ± 1.22 % and HSI: 8.773 ± 0.325 % vs 6.88 ± 1.19 % for control and nucleotide respectively). The histological examination of the gonads revealed that one of the nucleotide females was sexually immature and had not spawned (this was taken into account also for the relative fecundity calculations). The data from this fish were then removed and the calculations redone (Table 2. 8). When the data were analysed again, differences in the mean gonadosomatic index (C: 1.6840 ± 0.497 % vs NT: 3.00 ± 1.35 %) remained non-significant ($p = 0.595$). However, the HSI was significantly lower with the nucleotide diet (C: 8.773 ± 0.325 % vs. NT: 6.18 ± 1.14 %; $F = 6.56$, $p = 0.026$)(Table 2. 8).

Table 2. 8 Haddock broodstock condition and weight loss at the end of the 2002 and 2003 spawning seasons. Significant differences between diets are denoted by *. (Mean ± SEM).

Year	Parameters	Control	Nucleotide
2002	K-condition (g / cm^3)	1.3988 ± 0.0249	1.3918 ± 0.0296
	Weight loss (g)	229.1 ± 34.4	226.43 ± 8.36
2003	K-condition (g / cm^3)	1.3761 ± 0.0553	1.3280 ± 0.0542
	Weight loss (g) in ♀	204.8 ± 61.2	152.5 ± 60.8
	in ♂	17.1 ± 45.3	45.4 ± 11.4

GSI (%)	1.684 ± 0.497	3.00 ± 1.35
HSI (%)	8.773 ± 0.325	6.180 ± 1.140 *

2.4.4 Atresia studies

2.4.4.1 Atlantic halibut

Since it takes 7 – 8 years for Atlantic halibut to reach maturity and become broodstock, and also because their economic value is so high, no fish were killed at the end of the halibut trial. However, due to problems with water quality, two weeks after the spawning finished all the individuals from one of the nucleotide tanks died. Their ovaries were also sampled (§ 2.3.5.1) and atresia levels examined in the five females. No atresia was reported at all.

2.4.4.2 Haddock

As explained above, all the ovaries of the remaining females were extracted and the levels of atresia studied. These are summarised in Table 2. 9.

Table 2. 9 Atresia levels in the ovaries of haddock females fed both dietary treatments at the end of the second spawning season. Female 027 (in italics) was sexually immature.

Diet	Tank	Tag No.	% atretic oocytes	H S I (%)	G S I (%)
Control	F1	369	1.34	9.2	1.2
Control	F1	607	0.04		1.55
Control	F1	016	0.08	10	0.83
Control	F1	867	0.00		
Control	F3	791	0.87	9.21	0.87
Control	F3	584	3.31	8.62	0.81
Control	F3	802	0.14	7.35	0.68
Control	F3	805	3.25	7.17	0.97
Control	R2	579	62.82	7.68	6.35
Control	R2	046	8.74	9.88	0.75
Control	R2	315	0.00	9.39	2.20
Control	R2	527	0.00	9.23	2.28
Nucleotide	F2	833	2.62	6.42	1.17
Nucleotide	*F2*	*027*	*0.00*	*11.07*	*0.025*
Nucleotide	F2	612	14.67	6.14	4.2
Nucleotide	F2	889	n.q.	6.43	2.06
Nucleotide	R1	349	0.00	4.33	9.17

Nucleotide	R1	604	0.00	2.71	0.90
Nucleotide	R1	265	0.00	11.04	0.49

n.q. Not quantified, ovarian tissue too broken, although atretic oocytes were present.

Mean percentage of atretic oocytes in the control females was 6.72 ± 5.15, versus 2.88 ± 2.40 in the nucleotide females. However differences were not significant (W = 125.0; p = 0.311). There was a large variation in atresia levels between fish, ranging from 0 to 63% in the control group (one female with massive atresia, 62.82%) and to 15% in the nucleotide group. Only three out of seven nucleotide females had symptoms of atresia in their ovaries, whereas nine out of twelve females from the control group had some atresia. As a result of this, the incidence of atresia in the control females was 75% compared to 43% in the nucleotide group.

2.5 Discussion

Many studies involving nucleotide supplementation have been published in the last twenty years. Most of them however, involved small mammals or humans but few have involved fish species. Most of these experiments have looked at the effects that nucleotide supplementation had on different aspects of the immune system, gastrointestinal tract, or lipid metabolism but up until now the potential effects of these compounds on reproduction have not been targeted. The influence of nucleotide supplementation on feed consumption, fish condition and spawning performance will be discussed in this section.

2.5.1 Feed consumption & fish condition

The effects of nucleotide supplementation on the feed intake of Atlantic halibut and haddock were evaluated in the present study by comparing the feed consumption of two groups of fish, one fed a nucleotide-enriched diet and the other a control diet.

Dietary nucleotides have been reported to reduce feed intake in rats, dogs and chickens (Clifford & Story, 1976; Giesecke *et al.*, 1982; Brule *et al.*, 1988; Kubota & Karasawa, 1994; 1997). However, other studies have demonstrated the effectiveness of nucleotides as feed stimulants in different fish species (Ishida & Hidaka, 1987; Ikeda *et al.*, 1991; Kubitza *et al.*, 1997). In the present study, the enrichment of halibut and haddock broodstock diets did not affect feed intake. Similarly, sea bass (*Dicentrarchus labrax*) feed intake was unaffected when given increasing levels of RNA extract (Peres & Oliva-Teles, 2003). However, Tacon and Cooke (1980) observed feed intake and growth rates of rainbow trout fed diets with 10 % bacterial-RNA extract to decrease, while the incorporation of 0.6 – 4.1 % yeast-RNA increased significantly feed intake and weight gain in the same species (Rumsey *et al.*, 1992). These discrepancies may, at least in part, be explained by the different sources of nucleotides and the different nucleotide composition used in the different trials. Ikeda *et al.* (1991) demonstrated that from all the nucleotides tested, only inosine 5'-monophosphate (IMP), guanosine 5'-monophosphate (GMP), uridine 5'-monophosphate (UMP), UDP and UTP were effective as feeding stimulants while hipoxanthine, nucleosides (inosine, adenosine, guanosine, uridine), adenine 5'-monophosphate (AMP), ADP, ATP, IDP, ITP, GDP, GTP, xanthosine 5'-monophosphate, 3'-IMP and 3'-UMP were all ineffective. Something similar happens with the single bases: guanine and xanthine significantly increase feed intake while adenine decreases it (Rumsey *et al.*, 1992).

Feed consumption in halibut and haddock showed a normal pattern affected by their reproductive cycle. Sexually mature individuals from both species were observed to lose their appetite prior to, and during the spawning period as in other studies (Björnsson, 1995). Haddock accepted small amounts of feed during spawning as reported by Hislop *et al.* (1978). The mechanism responsible for reducing food ingestion during spawning is unclear, but also affects wild fish (Link & Burnett, 2001). It may be related to the swollen state that females exhibit when eggs in the ovary become hydrated, restricting the space of the body cavity and consequently the volume of food that can be ingested. Other factors such as hormone levels are likely to play some kind of role in regulating appetite. Feeding increased and reached normal

levels at the end of each spawning season in both species. The enhancement of halibut and haddock diets with nucleotides did not affect the feed intake in the two species tested during the three years of trials.

As mentioned above, Atlantic halibut and haddock reduced their feed intake as the spawning season approached. This is usually associated with weight loss and impoverishment of fish condition. It was expected that the nucleotide supplementation in broodstock feeds would lead to an improvement in broodstock condition at the end of the spawning season. However, it did not seem to be affected by the nucleotide-enriched diet in either of the two species tested (Section 2.4.3 & Table 2. 8). Tacon and Cooke (1980) demonstrated decreased growth rates when trout were supplemented with 10% bacterial RNA extract, in contrast to the improvements reported by Rumsey *et al.* (1992) and Adamek *et al.* (1996). Burrells *et al.* (2001b) described that the typical growth depressions seen in fish after vaccination were not observed in fish fed a nucleotide enriched diet. More recently Peres and Oliva-Teles (2003) used dietary RNA as a protein replacement rather than a functional additive and a 12% RNA extract reduced the growth rates. In the present study RNA levels in the feeds would only be around 0.01 % (Burrells pers. com.). In our study, weight loss during the spawning season did not differ between diets (Table 2. 8).

2.5.2 Spawning performance, HSI, GSI & atresia

The effects of nucleotide supplementation on spawning performance was evaluated in the present study by comparing various parameters of the control-fed group and the nucleotide ones in both fish species. The spawning periods of both species maintained under normal photoperiod conditions extended from March to May-June of each year (halibut: 2001; haddock: 2002-2003). Egg production followed a dome-shaped curve for halibut (Figure 2. 7) and haddock during the first spawning season (Figure 2. 12). However, this pattern was not observed in all the tanks during the second season and egg production decreased with time in

some tanks (Figure 2. 13). Hislop (1988) and Haug (1990) reported haddock and halibut to spawn during the first six months of the year. However, spawning time depends on latitudinal differences and halibut broodstock tend to spawn earlier at northern areas of Norway when compared to that from Shetland (Kjørsvik *et al.*, 1987; Haug, 1990).

One important aspect that must be taken into account when stocking groups of adult fish and collecting the eggs from an egg collector, as in haddock, is that the parentage of the offspring cannot be determined as different females may spawn on the same day. Therefore, egg production and relative fecundity could not be calculated on an individual-basis in the present study on haddock. Total relative fecundity of Atlantic halibut on both diets was of the same order of magnitude as that in previous studies also using Atlantic halibut (e.g. Norberg *et al.* 1991; Mazorra *et al.* 2003), although significantly higher in the nucleotide group (Table 2. 4). Norberg *et al.* (1991) remarked that this parameter is lower in first-time spawners compared to repeated ones. This could not be observed in our study because, although halibut first-year spawners were used, the effect of nucleotide supplementation on halibut was only monitored for one spawning season (2001). With regard to haddock, the total relative fecundity was not significantly different (Table 2. 7) between both diets, but higher in 2002 when compared to 2003 (Figure 2. 15). The relative fecundity per batch varied greatly over the spawning season in both years (Figure 2. 12, Figure 2. 13) ranging from 510 to 88,580 eggs/kg in 2002 and 500 to 140,200 eggs/kg in 2003. This phenomenon was also noted by Trippel *et al.* (1998) using mated pairs of haddock broodstock (range 4,000 – 108,000 eggs/kg). Nevertheless, the mean value was always higher in the nucleotide group, significantly in 2002, although not in 2003 (Figure 2. 16).

The nucleotide supplementation enhanced the relative fecundity of halibut and the batch relative fecundity of haddock, at least in the first spawning season of the latter. This is the first study to investigate the effects of nucleotides on reproductive success. The mechanisms by which nucleotide supplementation of broodstock diet affects fecundity are unknown. As mentioned by Izquierdo *et al.* (2001), changes in fecundity, reported in several marine species, could be

caused either by the influence of a nutrient on the brain-pituitary-gonad endocrine system or by alteration of the availability of a biochemical component during oogenesis.

Oogenesis is a process in which new cells are rapidly forming with concomitant intense DNA replication (leptotene) and RNA synthesis. In fish, although there is little information available, a period of intense RNA synthesis occurs during the initial stages of primary oocyte growth – previtellogenic growth- (Wallace & Selman, 1990). For instance, in rainbow trout the oocyte content of certain mRNA, such as those of vitellogenin (VTG) receptors and VTG processing enzymes, peak during previtellogenesis and at the onset of vitellogenesis (Prat *et al.*, 1998; Perazzolo *et al.*, 1999). Furthermore, there is evidence in zebrafish of mRNA transcripts passing from the maternal circulation into the egg and influencing embryo development (e.g. Goutel *et al.*, 2000). Nucleotides are synthesised endogenously *de novo* in tissues such as the liver. This is an energy-expensive process however, and the salvage route is preferably utilised when there is an exogenous source, such as diet, of nucleotides, nucleosides or bases. Nucleotides become essential when *de novo* synthesis is not able to fulfil the body's needs during periods of food deficiency, rapid growth (intensive cell division) and immunological stress (Uauy, 1989; Carver, 1999). Thus, tissues with a rapid cell turnover (such as the intestine, bone marrow and immune system) require exogenous nucleotides for synthesis of nucleic acids (Navarro *et al.*, 1996). Increased levels of DNA/RNA have been reported as a consequence of dietary nucleotides (Nuñez *et al.*, 1990; Uauy *et al.*, 1990; Yamauchi *et al.*, 1998; Tsujinaka *et al.*, 1999). Although no studies have been carried out, it is likely that this is also occurring in ovarian tissues during oogenesis. Since there is a higher requirement for nucleotides during intense cell division processes, the external supply of nucleotides through the broodstock diet may increase the availability of nucleic acids, having beneficial effects on oogenesis and thus enhancing the fecundity in the present study.

The fact that nucleotides participate in all major metabolic pathways suggests that these compounds may influence, directly or indirectly, fish reproduction at different levels. For instance, since water-soluble vitamins such as thiamin and riboflavin are derived from

nucleotide compounds, it could be suggested that nucleotides might affect the synthesis of the active forms of these vitamins. Fisher *et al.* (1996) presented the first evidence of a vitamin (thiamin) deficiency causing reproductive failure in fish, particularly Atlantic salmon (*Salmo salar*) and lake trout (*Salvelinus namaycush*). Other vitamins such as α –tocopherol and ascorbic acid affect also fecundity (Blom & Dabrowski, 1995; Izquierdo & Fernández-Palacios, 1997; Fernández-Palacios *et al.*, 1998).

Other nutrients are known to be affected by dietary nucleotides. Many studies have demonstrated that dietary nucleotides affect lipid metabolism in mammals and humans (Carver & Walker, 1995). For example, increases of long chain PUFA (polyunsaturated fatty acids) have been mentioned as a result of nucleotide supplementation (Gil *et al.*, 1986; Sato *et al.*, 1995). These PUFAs are greatly known to affect, directly or through their metabolites, fish reproduction. PUFAs regulate eicosanoid production, particularly prostaglandins, which are involved in several reproductive processes (Moore, 1995), including the production of steroid hormones and gonadal development such as ovulation (Mustafa & Srivastava, 1989). An n – 3 HUFA deficiency in the broodstock diet had negative effects on gilthead seabream fecundity (Almansa *et al.*, 1999) while increasing their levels in the diet of Japanese flounder (*Paralichthys olivaceus*), tended to increase it (Furuita *et al.*, 2000; Furuita *et al.*, 2002). However, despite the requirements of fish for EFA in their feeds, no studies assessing the effects of nucleotides on fish lipid metabolism have been carried out.

In addition, another hypothesis is the influence of nucleotides on gene expression. This has been suggested by Sanderson & He (1994), Tanaka *et al.* (1996) and Perez *et al.* (2002) in rat cells. Sanchez-Pozo & Gil (2002) demonstrated that dietary nucleotides can modulate gene expression by interacting with specific transcription factors, a phenomenon also confirmed in fish by Low *et al.* (2003). Nucleotides could modulate the gene expression of proteins involved in any aspect of gonad maturation and/or oogenesis.

In fish, lipids are mobilised from the liver into the gonads during oocyte maturation hence gonad weight and GSI increase while liver weight and HSI decrease (Kjesbu *et al.*, 1991). The liver of wild haddock represents approximately 5.2 % of the total weight; however cultured haddock have abnormally higher hepatic lipid deposition when fed moderate level of lipids in their diets. This has been associated with a low lipidic transport from the predominant storage (liver) to catabolic sites (muscle), due to low levels of very low density lipoprotein (VLDL) (Lall & Nanton, 2003). In the present study, the HSI of fish from the nucleotide group was lowered and closer to values found in wild fish (Table 2. 8). Deprivation of dietary nucleotides are known to have detrimental effects on the ultrastructure and function of liver (López-Navarro *et al.*, 1996a, b; 1997) as well as to induce lipid accumulation in rat hepatocytes (Carver, 1994; López-Navarro *et al.*, 1996a). Sánchez Pozo *et al.* (1986) indicated that nucleotide supplementation of baby formulae enhanced hepatic synthesis of lipoproteins in infants. This was later explained (Sánchez-Pozo *et al.*, 1994) as a consequence of the increment in apolipoproteins (apo) -the protein component of the lipoproteins-, and in particular apo A-I in HDL and apo B-100 in VLDL and LDL (Morillas *et al.*, 1994). Moreover, Sánchez-Pozo *et al.* (1995a,b) also reported that infants receiving nucleotides had higher plasma levels of apo A-IV and activities of lecithin-cholesterol-acyl-transferase (LCAT), a key enzyme in the lipoprotein metabolism. Fish liver synthesises VLDL and vitellogenin (VTG), a very high density lipoprotein, from which yolk protein precursors and lipids are derived via enzymatic cleavage (Tyler & Sumpter, 1996; Wiegand, 1996; Yeong Kwon *et al.*, 2001). Fish lipoproteins also consist of several apoproteins such as apo A-I-, A-II-, B-, C-, and E-like proteins similar and functionally equivalent to those of humans (Chapman, 1980; Babin & Vernier, 1989; Babin *et al.*, 1997). The addition of nucleotides into the broodstock diet may have enhanced hepatic lipid mobilisation through enhancing the synthesis of apolipoprotein of VTG and/or other lipoproteins and hence, although the HSI from both groups were probably reduced as a result of vitellogenesis, that of the NT group was significantly lower than the control one. Enhancing lipid mobilisation may have explained the findings from Adamek *et al.* (1996) in which nucleotides were found to decrease the fat content in flesh of rainbow trout. Studying the histo-morphological characteristics of fish liver was far beyond the initial objectives of this project

and data regarding possible changes in the hepatic histology are unfortunately not available. More research is required in this field, since no studies have looked at these effects in fish. Recently, Peres and Oliva-Teles (2003) reported the HSI of juvenile seabass to be increased, although dietary RNA was used instead of nucleotides and in concentrations 75 to 150 times higher than those in our experiment.

Regarding GSI, as explained above only female haddock were culled at the end of the spawning season and although GSI was assessed no statistical difference between control and nucleotide diets was found (Table 2. 8). These data are similar to those found in wild haddock (Clay, 1989; Waiwood & Buzeta, 1989). These authors indicated that haddock ovaries start increasing in size from November-December, peak around March-April (GSI = 6-11%), when the spawning season begins and then decrease from May to July (GSI= 0.3-1.9%), when the lowest levels are reached. Apart from ripe ovaries being much bigger than testes, another important difference between males and females is the pattern of gonad weight loss during the spawning season. Ovaries tend to maintain their weight during the season whereas testes gradually lose it, suggesting that the loss of spawned eggs is replaced up by the hydration of new ones (Clay, 1989). In our study, all the fish had just finished spawning so the GSI was decreasing as expected and in accordance with the previous studies mentioned above. Histological examination of the ovaries showed the presence of empty follicles confirming that all the fish had spawned, apart from one immature female (Table 2. 9). It was also noted that early previtellogenic oocytes were already present at this stage in most ovaries. These groups of yolkless oocytes have been referred to as nests and would suggest a two-year minimum intra-ovarian period from the time when oocytes were histologically recognisable until they were spawned (Raitt (1933), cited by Clay (1989)).

Atresia is a rapid process (Hunter & Macewicz, 1985; Kjesbu *et al.*, 1991) present in all stages of oocyte development, although the number of atretic oocytes increases at the end of the spawning period (Hunter & Macewicz, 1985). In this project, levels of atresia were not significantly different between dietary treatments and below 10% in both cases. However, there

was a large inter-female variation (Table 2. 9). The incidence of atresia seemed to vary between both diets: 9 out of 12 control females showed evidence of atresia while only 3 of the 7 nucleotide females presented atresia. However, from those nine atretic ovaries, the level of atresia was below 1% in four of them and above 10% (62.82%) in one fish. Atresia is a normal phenomenon in teleosts and as stated by Bromley *et al.* (2000) "*a low level of atresia might be a means of fine-tuning the size of the egg batches spawned, or removing damaged or abnormally developing oocytes*". Therefore, those four control females with atretic oocytes could be regarded as having "normal" levels of atresia. The reason for the massive atresia (>50%) in one of the control females is unknown, but it also happens in cod (Rideout *et al.*, 2000). Various factors such as hypophysectomy, administration of steroid hormones, biocides, temperature change and feed ration (Guraya, 1986) have been described as causing ovarian atresia in teleosts. Feeding at suboptimal levels may lead to malnutrition which has been related to increasing levels of ovarian atresia, causing the actual fecundity to be reduced in several species (Kjesbu *et al.*, 1991; Ma *et al.*, 1998; Rideout *et al.*, 2000; Macchi *et al.*, 2004). However in the present study feeding was not altered by nucleotide supplementation.

Chapter 3: Egg quality and physical parameters

3.1 Introduction to egg quality

Variation in egg quality is a limiting factor for the successful production of larval fish both in the wild and under cultivation. Therefore egg quality and the factors influencing it have received increasing attention in the last 10 years. The term 'egg quality' has been defined and used in various ways by different authors. Egg quality was defined by Kjørsvik *et al.* (1990) as "*the egg's potential to produce viable fry*". Several physical, genetic and chemical parameters, as well as the initial physiological processes occurring in the egg determine this potential to produce viable fry. If one of the egg characteristics which determine its capacity to survive is lacking, or is incomplete, egg development will fail at some stage. Kjørsvik *et al.* (1990), Bromage (1995) and Brooks *et al.* (1997) have reviewed egg quality in fish. The latest findings related to indicators and factors influencing egg quality in fish will be introduced below.

3.1.1 Factors affecting egg quality

Many factors affect egg quality and these have been reviewed by Kjørsvik *et al.*, (1990); Bromage, (1995) and Brooks *et al.* (1997). Key husbandry factors that are likely to have a major effect on egg quality are: egg overripening, bacterial colonisation of eggs, stress level of the broodfish and fertilisation practices adopted (Bromage *et al.*, 1994). See Figure 3. 1 for a summary of the suggested factors that influence egg quality.

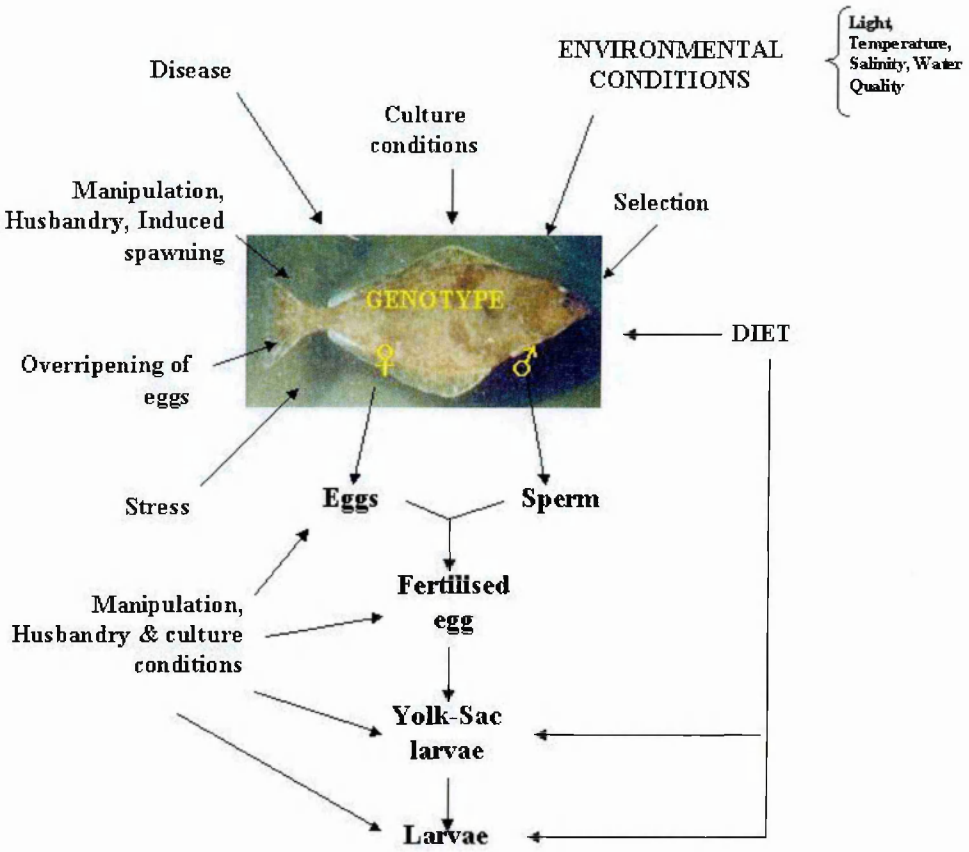


Figure 3. 1 Summary of suggested egg and larval quality determinants. Adapted from Bromage, 1995.

Overripening

Following ovulation unfertilised eggs undergo a process of ageing commonly known as overripening, which involves a series of morphological and compositional changes in the egg as well as a progressive loss of its quality or viability (McEvoy, 1984). Furthermore, Aegerter & Jalabert (2004) have demonstrated that post-ovulatory ageing of rainbow trout eggs is associated with an increased occurrence of morphological abnormalities. The duration of this optimum period of ripeness is species-dependent and also varies with the temperature (Table 3. 1). Overripening is often characterised as a discoloration or lack of transparency, fusion of cortical alveoli and a "dimpled" appearance of the cytoplasm (Kjørsvik *et al.*, 1990). There are very few studies available that have looked at the chemical changes occurring during overripening, but Rime *et al.* (2004) reported a strong accumulation of protein fragments in the ovarian fluid of rainbow trout during overripening and suggested a leakage of these compounds from the oocyte into the ovarian fluid during the postovulatory ageing process, and subsequently the use of these protein fragments as markers of egg quality.

Table 3. 1 Period of viability and optimum egg quality before overripening in different fish species. Compiled from Kjørsvik *et al.*, (1990) and Bromage 1995

Common name	Species	Time	Temp (°C)	Reference
Arctic charr	*Salvelinus alpinus*	7 days	6.5	Krieger & Olson (1989)
		5 days		Gillet (1991)
Black catfish	*Rhamdia sapo*	5-9 h		Espinach Ros *et al.* (1984)
Asian catfish	*Pangasius hypophthalmus*	2 h	28-29	Legendre *et al.* (2000)
Brown trout	*Salmo trutta*	<28 h	15	Billard & Gillet (1981)
		>76 h	10	
Catfish	*Clarias macrocephalus*	10 h	26-31	Mollah & Tan (1983)
Coho salmon	*Oncorhynchus kisutch*	20 days		Fitzpatrick *et al.* (1987)
Goldfish	*Carassius auratus*	24 h	23	Formacion *et al.* (1993)
Nile tilapia	*Oreochromis niloticus*	1 h	28	Rana (1990)
Rainbow trout	*Oncorhynchus mykiss*	10 days		Nomura *et al.* (1974)
		5-7 days	10-12	Sakai *et al.* (1975)
		4-6 days	10	Springate *et al.* (1984)
Ayu	*Plecoglossus altivelis*	24 h		Hirose *et al.* (1977)
Atlantic halibut	*Hippoglossus hippoglossus*	4-6 h	4-5	Bromage *et al.* 1994
				Homelfjord (1991)
				Norberg *et al.* (1991)
Cod	*Gadus morhua*	9 h	5	Kjørsvik & Lønning (1983)
Herring	*Clupea harengus*	14 days	8-10	Hay (1986)
Japanese flounder	*Paralichthys olivaceus*	2-3 days	12	Hirose *et al.* (1977)
Murray cod	*Maccullochela peeli*	2-3 h	20	Rowland (1988)
Sabalo	*Prochilodus platensis*	1 h	25-27	Fortuny *et al.* (1988)
Stripped bass	*Roccus saxatilis*	1 h		Stevens (1966)
Turbot	*Scophthalmus maximus*	10 h	12-14	McEvoy (1984)
				Howell & Scott (1989)
Weather loach	*Misgurnus anguilicaudatus*	3-8 h		Suzuki (1975)

Bacterial colonisation

After fertilisation, eggs are incubated and mortalities occur. Moribund and dead eggs become colonised with bacteria and/or fungus and if they are not removed quickly from the incubation system, viable eggs may become infected. Removal of dead eggs together with incubating eggs in high quality water will enhance egg survival (Bromage, 1995).

Nutritional and environmental conditions

Ideally broodstock should be maintained under controlled conditions matching as far as possible or improving on those to which they would have been exposed in the wild. However, in practise it is difficult to manage all the rearing conditions and comparisons of wild and captive broodstock usually report that the egg quality is higher in wild fish compared to captive stocks. The superior quality of wild eggs is believed to be largely a function of nutritional and

environmental factors. The former has been described in the "effects of nutrition on egg quality" of section 2.1.1. Environmental factors that may affect egg quality include light conditions and the physico-chemical conditions of the water (temperature, salinity, pH, etc.) in which broodstock are maintained and eggs are incubated (Figure 3. 1). Water temperatures during spawning and egg incubation are particularly important for egg quality since it affects metabolism, activity and structure of the developing embryo. It has been established that temperature alterations can induce changes at gene expression level and are likely to affect embryo and larval development (Moreau *et al.*, 1991; F. Venegas, M. Rojas & O. Goicoechea pers. comm.). Any disruption of the optimal environmental culture conditions will act as a stressor and affect fish reproduction, gamete quality and progeny (Schreck *et al.*, 2001) and this is well known by hatchery companies. The use of modified light or photoperiod regimes is a normal application for altering the rate of maturation and the time of spawning in fish cultivation (reviewed by Bromage *et al.*, 2001). Some studies have reported improved egg survival while in other the egg viability was reduced as a result of photoperiod manipulation; therefore, to what extent egg quality is affected by manipulation is not clear and it may depend on the time of the year when the advance/delay in spawning occurs (Brooks *et al.*, 1997).

Broodfish age

The age of the broodfish is an important factor to take into account. First year spawners do not perform as well as in later successive spawning seasons; consequently the quality of the eggs improves in the following spawning seasons (seabass: Carrillo *et al.*, 2000). The fundamental mechanisms underlying the ageing process are generally poorly understood, but one of its consequences is that eggs produced by old females are of very poor quality in terms of buoyancy, hatching rates and with high levels of aberrant developing embryos. In seabass, pubertal or ageing fish are avoided and only individuals between the second and fifth sexual cycle are chosen (Carrillo *et al.*, 1995; 2000).

Genetic influences

The genetic make-up of the fish provides the blueprint on which its performance is based and this includes the quality of the eggs. The importance of genetic influences on egg quality has been reviewed by Brooks *et al.* (1997). Apart from the genetic information contained in the chromosomes of unfertilised eggs, a few studies have confirmed the transfer of nucleic acid molecules from the parents into the oocytes. Maternal RNA supports oocyte protein synthesis during oogenesis and controls early embryonic development, until transcription of the embryonic RNA begins after fertilisation. There is little information on gene transcription and/or mRNA translation in fish oocytes/embryos; however the proteins synthesised and the mechanism controlling their expression are likely to play a central role in determining egg quality (Brooks *et al.*, 1997). In recent studies with seabass (Saillant *et al.*, 2001) a male effect was detected on hatching and it was suggested that might reflect a paternal genetic component determining survival from fertilization until hatching.

3.1.2 Egg Quality indicators

One of the major obstacles to studying egg quality in different species of fish is the difficulty in establishing parameters that produce reliable predictors of quality. Several parameters and methods have been proposed to determine egg quality. However, none of the parameters individually appear to be enough to fully characterise quality and hence a combination of them gives more satisfactory results. Moreover, not all the characteristics behave similarly in different fish species and they are not always consistent indicators of egg quality. Ideally, these predictors should be easily assessed, not require lengthy or sophisticated lab procedures and should be carried out soon after egg collection in order to avoid using unnecessary incubating facilities and staff time.

Fertilisation-related indicators

Fertilisation and hatching rate have been used as important criteria in most egg quality investigations. Both are useful parameters to detect poor egg quality and are used in broodstock nutrition studies. However, some studies have reported that fertilisation rate does not always correlate with good survival and development in later embryonic stages (see review by Kjørsvik *et al.* 1990). During fertilisation and activation, the cortical alveoli break down and start the formation of the perivitelline space. This process is correlated with egg quality and poor quality eggs have cortical alveoli present and less perivitelline space after fertilisation (Kjørsvik & Lønning, 1983; McEvoy, 1984).

Physical parameters

Physical and physiological characteristics have also been suggested as indicators of egg quality. Hardening of the chorion takes place after fertilisation and the egg strength, in terms of chorion hardness, is greater in good than in poor quality eggs of various species (Kjørsvik *et al.*, 1990). The increase in the relative egg weight during water hardening is correlated with viability and it has been used for egg quality control in salmonids and cyprinids (Lahnsteiner *et al.*, 1999; 2001; Lahnsteiner & Patzner, 2002). Chorion appearance is also used as a predictor of quality and wrinkled egg membranes or a golf-ball like surface both tend to predict poor egg viability in Atlantic halibut (*Hippoglossus hippoglossus*) (P. Smith, pers. comm.). Egg shape also tends to change in poor quality cod eggs (*Gadus morhua*) (Kjørsvik & Lønning, 1983) and becomes irregular. Many hatcheries culturing marine species distinguish "good" from "bad" eggs depending on their ability to float or sink in water respectively (McEvoy, 1984; Carrillo *et al.*, 1989; Kjørsvik *et al.*, 1990). However, changes in specific gravity and hence in buoyancy may partly be due to osmoregulatory variation in egg batches over the season as in sprat (*Sprattus sprattus*) (Nissling *et al.*, 2003). Furthermore, for a number of species, including halibut, no such correlation of buoyancy with quality has been consistently reported (Bromage *et al.*, 1994).

Morphological and chromosomal parameters

Egg morphological characteristics have also been studied as quality indicators. In fish eggs, the large yolk volume restricts the process of cleavage to a small area of cytoplasm at the animal pole and the large early blastomeres are easily visible in non-pigmented eggs. Blastomeres are undifferentiated cells that form the basis for the developing embryo; therefore a deviation (or defect) in these cells will influence the further development of the embryo more strongly than defects occurring in single cells at later stages of development. For most fish species, 'normal' early blastomeres are regular in size and shape. Cell morphology, also used in pollution studies and toxicological experiments, differs in abnormal eggs, including blastula-stage embryos, and do not complete embryogenesis (Westernhagen, 1988). The symmetry and morphology of early blastomeres appear to be a consistent early indicator of egg viability for several species such as herring (*Clupea harengus*), Dover sole (*Solea solea*), red seabream (*Pagrus major*), cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), Japanese flounder (*Paralichthys olivaceus*), Atlantic halibut and haddock (*Melanogrammus aeglefinus*) (Kjørsvik *et al.*, 1990, 2003; Bromage, 1995; Bromage *et al.*, 1994; Shields *et al.*, 1997; Vallin & Nissling, 1998; Rideout *et al.*, 2004). Other useful indicators of egg quality are shape and transparency as well as the number and distribution of lipid droplets, although the latter is species-dependent. 'Good' eggs are generally described as transparent and perfectly spherical with symmetrical early cleavages (Kjørsvik *et al.*, 1990).

Three types of chromosomal abnormalities are generally encountered when the morphology of chromosomes is assessed during the mitotic division of fish embryo cells: delayed division of some centromeres; fragments or parts of chromosomes remaining in the equatorial plane; and chromosomes that do not divide and remain in the equatorial plane (Kjørsvik *et al.*, 1990). Chromosomal morphology during the early stages of embryonic development might provide useful information regarding the viability of an egg batch since chromosomal errors may become lethal. Thus, clear correlations between egg viability and its cytogenetic status have been derived from toxicological studies and the monitoring of polluted areas (Klumpp & Westernhagen, 1995; Cameron & Westernhagen, 1997).

Ovarian fluid

Kjørsvik & Lønning (1983) reported differences in the ovarian fluid of cod in consistency, colour and volume, and cod producing normal eggs were likely to have a more transparent and 'liquid' ovarian fluid than those producing poor eggs. Measurements of ovarian fluid pH have been used to assess egg quality successfully in marine and freshwater fish species (Fauvel *et al.*, 1993; Lahnsteiner *et al.*, 1999; 2001). Lahnsteiner *et al.* (1999) reported that high levels of protein in the ovarian fluid indicated low egg quality in lake trout (*Salmo trutta lacustris*).

Egg size

Is bigger better? Historically in aquaculture there has been the perception that bigger eggs are better in terms of quality. However, there is no clear evidence suggesting that egg diameter is a good criterion for egg quality. Many studies have demonstrated that egg size may vary within species, between populations of the same species and also between individuals of the same population. Parental size affects egg size, with larger females producing larger eggs in many species such as Atlantic salmon (*Salmo salar*), rainbow trout and cod (Thorpe *et al.*, 1984; Bromage, 1995; Vallin & Nissling, 2000). A recent study by Pakkasmaa *et al.* (2001) showed that the final egg size is affected not only by the initial size of the oocyte but also by both the female and the male through the process of swelling. Egg size is also influenced by the nutritional status of female haddock and cod (Hislop *et al.*, 1978; Kjørsvik *et al.*, 1990) and in asynchronous spawners the size of the eggs decreases over the spawning season, a phenomenon associated with the decreasing resources of the female (Hsiao *et al.*, 1994).

It is known that egg and larval size is correlated. Larger larvae in the wild tend to survive longer during the first days of their life without food than those from smaller eggs (e.g. Blaxter & Hempel, 1963). However it has been shown that egg size has no direct implications for overall egg quality under more favourable conditions (reviewed by Kjørsvik *et al.*, 1990). Nissling *et al.* (1998) observed no effect of egg size on egg viability, whereas larval viability varied due to differences in egg size.

However, in the wild under special environmental conditions, egg size may be a factor of major importance for egg survival. For example, successful spawning of cod in the Baltic Sea is restricted to the deep basins where oxygen deficient conditions prevail. Since egg buoyancy is positively correlated to egg size, the vertical distribution is highly influenced by egg size (Kjesbu *et al.*, 1992; Nissling *et al.*, 1994) and therefore egg survival in the Baltic is highly influenced by egg size (Nissling *et al.*, 1994).

Biochemical composition of eggs

In oviparous fish species, the composition of fish eggs is largely influenced by broodstock nutrition. Certain components are known to be essential for embryonic development and they have to be present in certain amounts to satisfy the biological demands of the developing embryo. As suggested by several authors (e.g. Fernández-Palacios *et al.*, 1995), the chemical composition of fish eggs is related to their quality since egg composition must satisfy embryonic nutritional needs for development and growth. Therefore, biochemical analysis of these compounds might be used as egg quality parameters. Many biochemical parameters have been suggested as egg quality indicators, however sometimes these are species-dependent and while they are described as an indicator of 'good eggs' for one species, they may not be for a different species. This is due to the fact that the nutritional requirements of fish vary between species.

The importance of pigments, and in particular carotenoids, on egg quality in salmonids was reviewed by Craik (1985). In marine species, the carotenoid content of yellowtail (*Seriola quinqueradiata*) eggs was greatly affected by broodstock diet, and eggs with a stronger yellowish colour and with a high content of zeaxanthin and lutein also showed a better quality (Verakunpiriya *et al.*, 1996). However, striped jack eggs do not incorporate carotenoids into their eggs (Vassallo-Agius *et al.*, 2001c); hence its abundance is not a relevant egg quality marker for this species.

Kjørsvik *et al.* (1990) discussed vitamin C (ascorbic acid) as an egg quality predictor. Sandnes *et al.* (1984) found a direct correlation between hatching rate and ascorbic acid content in rainbow trout (*Oncorhynchus mykiss*). Recent experiments have confirmed that the antioxidant function of vitamin C in the gametogenesis of teleost fish is critical for the fertilising ability of sperm and eggs and more specifically to maintain the DNA integrity of the gametes (Dabrowski & Ciereszko, 2001). The use of vitamin C as a quality marker of eggs might then be suggested, although more investigation is needed to confirm it.

Large amounts of essential fatty acids (EFA) are required during embryonic and larval development (Sargent, 1995) and many nutritional studies have evaluated the effects of altering the lipid content of broodstock diet on egg quality. Therefore, lipids have also been suggested as indicators of egg quality. Fernández-Palacios *et al.* (1995) reported that the n – 3 HUFA content of the eggs should not be used as a sole criterion to determine egg quality of gilthead seabream (*Sparus aurata*) and in a study carried out two years later (Fernández-Palacios *et al.*, 1997) n – 9 fatty acid content of eggs was highly positively correlated with egg viability and hatching rate. Data from Navas *et al.* (1997) showed a clear link between DHA/EPA ratio and egg quality, in terms of egg viability (floating fraction) and hatching rates in sea bass. Furthermore, DHA/EPA and ARA/EPA ratios of seabass (*Dicentrarchus labrax*) diets were reported to correlate with the same ratios of eggs (Bell *et al.*, 1997), which were also correlated with higher hatching rates (Navas *et al.*, 2001). Bell (1998) suggested that while the optimum ratio of DHA/EPA/AA in broodstock diets is likely to be species specific, the importance of a high AA/EPA ratio in eggs may be ubiquitous. This has been also confirmed in later studies (Bruce *et al.*, 1999; Asturiano *et al.*, 2001; Navas *et al.*, 2001). More efforts should be directed toward establishing the optimum ratio DHA/EPA/ARA in broodstock diets and consequently in eggs in order to use it as a predictor of quality.

The activities of certain enzymes present inside the egg have also been suggested as biochemical markers of quality: Lahnsteiner *et al.* (1999) reported that the determination of biochemical egg compounds such as NADH/NAD ratio, malate dehydrogenase activity and

respiration rate of eggs gave reliable information on egg viability in lake trout (*Salmo trutta lacustris*). Fertilisation rate also correlated with the activities of malate dehydrogenase and pyruvate kinase (enzymes that are involved in cell energy metabolism) in cyprinid eggs (Lahnsteiner *et al.*, 2001). Srivastava and Brown (1991) suggested the energy content of eggs as an egg quality marker since it was associated with fertilization and hatching success in Atlantic salmon (*Salmo salar*).

Regarding nucleotides, energy charge[1] (EC) is expressed as a measure of the adenine nucleotide pool (Atkinson & Walton, 1967). The EC value of organisms in normal condition is usually between 0.84 and 0.95 and values below 0.80 have generally been interpreted as indicative of metabolic stress (Atkinson, 1977). In some studies on red drum (Vetter *et al.*, 1983), the EC ratio showed a decline from 0.87 after fertilisation to 0.60 at the end of the yolk-sac larvae stage. Furthermore Bouleckbache *et al.* (1989), in a study on carp (*Cyprinus carpio*) oocytes reported highest mortality rates of embryos when both ATP (adenine triphosphate) and EC values reached their lowest level. It is therefore suggested that EC could also be linked to egg-quality. Recently, DNA/RNA ratio levels were not correlated with egg viability (Lahnsteiner *et al.*, 2001) although these are helpful in estimating the growth rates of embryos and larvae (Clemmesen, 1988 cited by Lahnsteiner *et al.*, 2001).

3.2 Aims

The aim of this chapter was to assess the potential benefits of the nucleotide diets on the quality and physical characteristics of the eggs produced by Atlantic halibut and haddock broodstock throughout 2001 and 2002-2003 respectively. Fertilisation rate, hatching rate and blastomere morphology were used as egg quality parameters, whereas egg physical features included dry weight, wet weight, specific gravity and egg diameter.

[1] $EC = \frac{[ATP] + 0.5 \times [ADP]}{[ATP] + [ADP] + [AMP]}$ (Atkinson & Watson, 1967)

3.3 Material and Methods

All the protocols followed to assess egg parameters are described in this section. Egg incubation procedures, egg quality assessment and physical properties of eggs from halibut and haddock broodstock are included.

3.3.1 Halibut

3.3.1.1 Egg Incubation

While egg quality parameters used for halibut and haddock are basically the same, incubation systems differ slightly and will be described below.

3.3.1.1.1 System Description

The egg incubators consisted of 70-L black polyethylene cylindrico-conical 'Paxton' tanks. The water inlet was located at the bottom of the incubator via a T-connector to the central drain, and a 500 μm screen net was submerged at the top of the incubator and connected to the overflow in order to prevent the eggs from draining out. The water inlet could also be used as a secondary outflow point during dead egg collection (§ 3.3.1.1.2). The incubator was covered to keep the eggs in darkness (Helvik & Walther, 1992). Two different sources of water were used, both UV-sterilised, 1μm filtered, at 5.5-6 °C and differing only in salinity: the main ambient source was at 32-34 ‰; and the second was at high salinity (40 ‰). The latter was premixed in a reservoir and used to remove dead eggs throughout the incubation period (§ 3.3.1.1.2).

3.3.1.1.2 *Husbandry Operations*

Husbandry operations were very similar to those described by Mangor-Jensen *et al.* (1998a). After fertilisation and rinsing (§ 2.2.2.1), eggs were transferred to the 70-L cylindro-conical polyethylene tanks at 5.5 – 6 °C and 35.5 ‰ salinity. Halibut eggs are transparent and neutrally buoyant in 32-34 ‰ salinity (Mangor-Jensen & Waiwood, 1995) so under high salinity conditions, fertilised eggs float to the top of the water column while dead and unfertilised eggs tend to sink and accumulate at the bottom of the tank. Twenty minutes after fertilisation, the incubator drain was opened and the first drop-out of non-viable eggs was collected in a soft net. Excess water was removed using absorbent paper and the drop-out weighed on a scale. Egg samples were taken in order to calculate the number of eggs per gram of drop-out; the number of eggs in the drop-out could then also be estimated. The same operation was carried out 24 hours after fertilisation to collect the remaining unfertilised and dead eggs. The number of non-viable eggs was also estimated in the second drop-out.

Upwelling flow in the incubator tanks was turned on 24 hours post-fertilisation at a flow rate of 2 L·min $^{-1}$, to maintain developing eggs in the water column. Ambient conditions were darkness, 5-6 °C and 34-36 ‰ salinity. Dead and non-viable eggs were removed from the incubator every 3-4 days, using a 'salt-plug' technique, described by Jelmert & Rabben (1987). The main water supply was turned off and high salinity water was instead used for the upwelling flow. The water (~ 40 ‰) was introduced at a flow rate as low as 0.20 L·min $^{-1}$ in order to prevent mixing of water. A halocline was therefore formed in the tank, separating a high salinity mass of water at the bottom (containing all the dead eggs) from a less saline water mass containing the buoyant developing eggs. The high salinity layer was drained and dead eggs collected in a soft net. The number of dead eggs was also estimated after removing excess water.

Sixty six egg batches were collected in total although only those with a fertilisation rate higher than 75 % were incubated. However, due to the reduced number of incubators available (12),

only 26 batches could be monitored until the end of the yolk sac stage (15 from the nucleotide and 11 from the control group). Egg incubation in the 'Paxton' tanks lasted 75-80 °days (degree days). After this time eggs hatched and larval samples were taken for dry weight, morphometric and nucleotide content analysis.

3.3.1.2 Egg quality assessment

Halibut egg quality was evaluated by using the following parameters: fertilisation rate, blastomere morphology and hatching rate.

a) **Fertilisation rate** (FR): FR was assessed at the 8-cell stage, 96 ° h (degree hours) after fertilisation. As explained above, fertilised eggs were transferred to 70-L cylindro-conical black polyethylene tanks. Thereafter, sub-samples of eggs from the floating fraction were taken from the tank and the numbers of developing (fertilised) and non-developing (unfertilised) eggs determined by the presence of dividing cells. Fertilisation rate was calculated according to the following formula:

$$FR = \frac{No.\,dev.\,eggs}{(No.\,dev.\,eggs + No.\,ndev.\,eggs)} \times 100 \quad \text{[Eq.3. 1]}$$

where:

FR is fertilisation rate expressed as percentage,

No. dev. eggs is the number of developing eggs.

No. ndev. eggs is the number of non-developing eggs.

b) Egg quality was also assessed by following a method described by Shields *et al.,* (1997) specifically for Atlantic halibut eggs. The procedure is based on the analysis of **blastomere morphology** (BM) 96 °-h (16 hours at 6 °C) after fertilisation, at the 8-cell stage of

development. The procedure is as follows: Fertilised eggs were randomly sampled at the 8-cell stage from the egg incubators. Sampled eggs were deposited in 96 well plates filled with 200 µl of UV-sterilised, 1 µm-filtered seawater (one egg per well) at 5.5-6.5 °C under dim red light. Once in place, each egg was examined under a binocular microscope and given a score from 1 to 4 ('abnormal' to 'normal') for each of the following parameters:

- Symmetry: bilateral symmetry along the axis of the 8 blastomeres. 'Normal' appearance is symmetrical.
- Cell size: uniformity of cell size between all 8 blastomeres. Cells of same size are 'normal'.
- Adhesion: proximity of adjacent cells. 'Normal' blastomeres have adjacent cells in contact.
- Margins: definition of cell margins. Good discreteness of cell margins is referred to as 'normal'.
- Inclusions: vacuole inclusions between blastomeres. 'Normal' means no inclusions.

The BM score for a batch of eggs was the mean of the sum of the scores for each egg (0-20). Once assessed, plates were then sealed and stored in a cool incubator at 5.5 °C and total darkness until hatch. At approximately 75 ° days, the plates were removed from the incubator and studied. Each egg was examined and the number and position of hatched eggs recorded. Hatching rates could be therefore estimated and related to BM score.

c) **Hatching rate** (HR): The microtiter plate technique was used previously with other marine species such as halibut (Shields *et al.,* 1997), sea bream and sea bass (Panini *et al.*, 2001). Hatching rate was calculated from the eggs incubated in plates according to this formula:

$$HR = \frac{no.\ hatched\ eggs}{no.\ fertilised\ eggs} \times 100 \qquad \textbf{[Eq.3. 2]}$$

3.3.1.3 Egg physical properties

Physical characteristics, such as wet and dry weight, volume and density of halibut eggs were also studied. **Volume** and **wet weight** of a single egg were estimated according to formulas 3.3 and 3.4 respectively.

$$EV = \frac{batch\ volume}{batch\ no.\ eggs} \quad \textbf{[Eq.3. 3]}$$

$$EM = \frac{batch\ mass}{batch\ no.\ eggs} \quad \textbf{[Eq.3. 4]}$$

where:

EV is the volume of a single egg; **EM** is the wet weight of a single egg,; 'batch volume' is the volume (L) of eggs collected in each batch; 'batch mass' is the mass (g) of eggs collected in each batch, and 'batch no. eggs' is the total number of eggs collected in each batch, estimated as described in section 2.3.2.

Halibut eggs were freeze vacuum dried and **dry weight** measured on a scale. **Water content** (WC), expressed as a percentage, was calculated according to the following equation:

$$WC = \frac{Wet\ weight - Dry\ weight}{Wet\ weight} \times 100 \quad \textbf{[Eq.3. 5]}$$

Egg density – specific gravity – was estimated by dividing EM, expressed in grams, by EV, expressed in millilitres (ml).

3.3.2 Haddock

3.3.2.1 Egg incubation

As explained in section 2.3.2.2, collected batches of haddock eggs were staged and while mixed batches were discarded, homogeneous batches (i.e. in which eggs were in a similar developmental stage) were incubated after disinfection.

3.3.2.1.1 System Description

Haddock egg incubators were also 70-L cylindro-conical 'Paxton' tanks in which the water inflow was located at the bottom of the tank from a narrow inlet pipe, and the outflow consisted of a central stand-pipe surrounded by a cylindrical 250 μm nylon mesh. Flow rates were 1.5 - 2 $L \cdot min^{-1}$. A perforated circular tube located around the base of the mesh and forming a collar was connected to an air line that provided continuous aeration. Water was UV-sterilised, 5 μm-filtered and of 32-34 ‰ salinity. Incubation temperature was 8-10 °C and illumination was kept at a low level (10 lux), except during brief periods of husbandry.

Due to the large number of egg batches collected, particularly during 2002 (six per day, i.e. one per tank) and the limited incubator availability, not all available batches were incubated. Instead, fifteen homogeneous egg batches (i.e. with the same developmental stage) from each treatment were incubated and followed until yolk absorption in 2002. Since the egg production in 2003 was much lower than the previous spawning season, only three egg batches from each dietary group were incubated and followed this time, until the beginning of first feeding.

3.3.2.1.2 Husbandry operations

Most egg mortalities occurred during the first 48 hours after stocking. Aeration and water inflow were switched off to ease the collection of dead eggs, which sank to the bottom of the tank, and 10-15 minutes later were easily siphoned off. These eggs were then weighed on a scale after removing excess water.

Incubated egg batches were left to hatch in the incubators (circa 110 ° days) and larval samples were collected for dry weight, morphometrics, survival and first feeding experiments (Chapter 4), and nucleotide analysis (Chapter 5).

3.3.2.2 Egg quality assessment

Egg quality of the batches was assessed by using fertilisation rate (FR) and hatching rate (HR). Morphology of the blastomeres could not be studied with haddock since eggs had already passed the 8-cell stage by collection, the optimum stage to check their morphological features.

a) **Fertilisation rate**: A subsample was taken from the floating fraction of each batch, placed in a petri dish and the percentage of fertilised eggs was estimated under a binocular microscope. This procedure was repeated three times for each batch and the mean value recorded as fertilisation rate.

b) **Hatching rate**: As with halibut, plates were used to assess the HR of haddock eggs. However, since eggs had already passed the 8-cell stage at the time of collection and BM could not be studied it was not necessary to follow each individual egg and therefore, 12-well plates were used instead. Thus, on the following day after collection, plates were prepared as follows: Fifty (50) µl of egg sample were collected by using a micropipette to which a tip with a cut end was attached. Eggs were then stocked in each well containing 2 ml of clean UV-sterilised, 1 µm filtered seawater at 8-9 °C and 34-35 ‰ salinity. Two well plates were prepared per egg batch. There were at least two eggs incubated in each well. Eggs were incubated in the plates at 8 °C and hatching rates checked at 110 ° days (degree-days).

3.3.2.3 Egg physical properties

Physical characteristics, such as wet and dry weight, volume and density of haddock eggs were studied over two consecutive spawning seasons (2002-2003).

a) **Egg wet weight** was calculated by removing excess water of ten eggs and weighing them on a scale. This operation was done in triplicate and the mean value recorded as egg wet weight for each batch.

b) **Egg dry weight**: Those eggs previously used to determine the wet weight were placed inside an eppendorf tube, freeze vacuum dried and dry weight measured on a scale. This operation was also done in triplicate.

c) **Water content** (%): Calculated according to Eq. 3.5

d) **Egg size**: Images of eggs were captured, while staging the egg batches, by using a digital camera mounted on a binocular microscope and connected to a computer. At a later stage egg diameter measurements were performed using the UTHSCSA *ImageTool* Version 2.03 software (University of Texas Health Science Center, San Antonio). Over five eggs were measured from each batch and the mean value was recorded as egg diameter for each batch. Egg volume could therefore be calculated for each batch.

e) **Egg density**: Relative density, also called specific gravity, of the eggs was calculated by dividing the mean wet weight by the mean egg volume for each batch.

3.3.3 Data analysis

Statistical analysis of the data was similar to that described in section 2.3.6, using parametric or non-parametric tests. ANOVA test, Man-Whitney test and Mood's tests were used to examine differences between both dietary treatments. ANCOVA test was used to study the effect of the diet on a parameter (e.g. egg size) that was suspected to be influenced by a covariate (e.g. date). Least squared means analysis was additionally used if the interaction diet-covariate (diet*covariate) was significant. General linear models (GLM) as defined previously (§ 2.3.6) were also used to take into account all the parameters that could influence the response of the variable and correlation and regression analysis to follow the relationship between variables.

3.4 Results

3.4.1 Halibut Trial

3.4.1.1 Egg quality

The effects of the nucleotide diet on the egg quality of Atlantic halibut were examined by comparing the fertilisation rates (FR), blastomere morphology index (BM) and hatching rates (HR) between control and nucleotide treatments.

Fertilisation rates

Fertilisation rates were very similar between both diets (control: 68.15 ± 4.92 % vs. nucleotide: 71.78 ± 3.77 %, F = 0.31, p = 0.549; Figure 3. 2). Since no significant difference was found between both diets and among tanks (F = 0.21, p = 0.955), data were pooled and the variation of the fertilisation rate was studied along the season. Fertilisation rate followed a dome-shape distribution along the season, with the highest values occurring at the middle of the spawning season (C: F = 5.60, p = 0.026, R^2 = 19 %, R^2_{adj} = 12.8 %; NT: F = 6.98, p = 0.003, R^2 = 31.8 %, R^2_{adj} = 27.2 %; Figure 3. 3). As explained in the previous chapter, high fertilisation rates led to low drop-out 24 h after fertilisation (r = -0.5574, p < 0.0001).

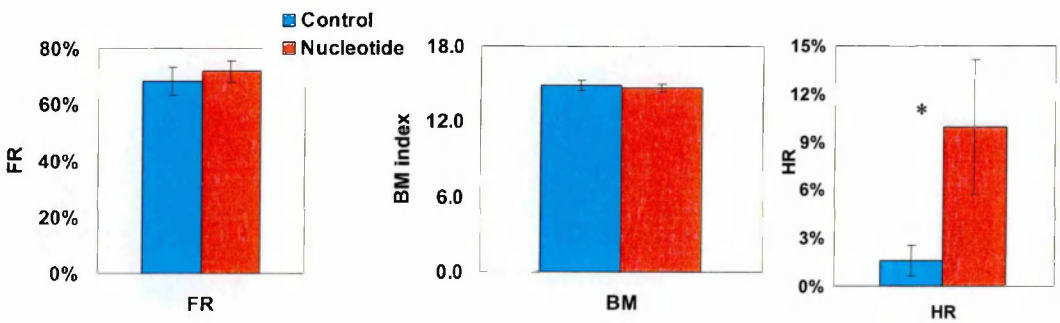


Figure 3. 2 Halibut egg quality parameters: Fertilisation rate (FR); Blastomere morphology (BM) and Hatching rate (HR). Significant difference is denoted by *. (Mean ± SEM).

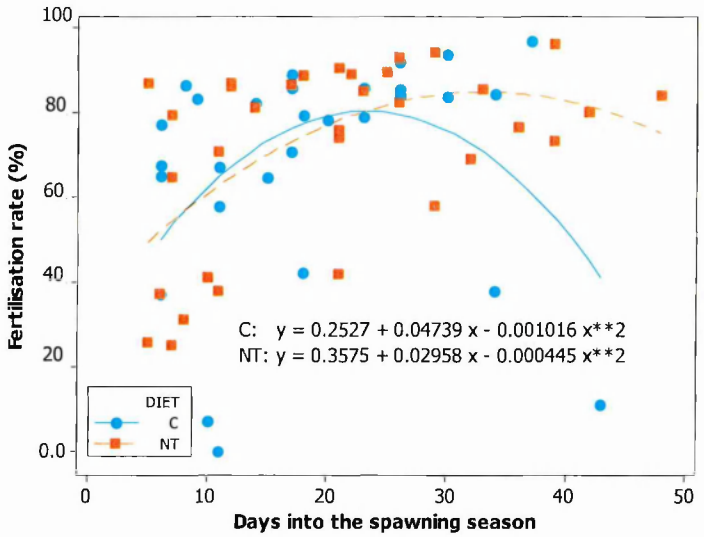


Figure 3. 3 Variation in fertilisation rate of Atlantic halibut over the season in the control (C: blue circles) and the nucleotide (NT: red squares) group.

Blastomere morphology (BM)

As explained by Shields *et al.* (1997), the BM score ranges from 0 (worst) to 20 (best). The BM index of the egg batches from the control group scored 14.84 ± 0.39 versus 14.62 ± 0.31 in the nucleotide treatment (Figure 3. 2) and differences were not significant ($F = 0.19$, $p = 0.661$). A general linear model analysis showed that blastomere morphology was significantly affected by date (Table 3. 2) and decreased over the spawning season ($r = - 0.3807$, $p = 0.0045$; Figure 3. 4).

Table 3. 2 General linear model analysis of blastomere morphology of Atlantic halibut fed both dietary treatments. ** Denotes significant effect.

	DF	Sum of Squares	Mean Square	F Value	P Value
Diet	1	0.1527	0.1527	0.07	0.717
Tank (Diet)	4	19.2179	4.8045	2.34	0.060
Female (Diet Tank)	14	44.1353	3.1525	1.54	0.109
Date	1	9.1984	9.1984	4.62	0.039**
Diet * Date	1	0.1528	0.1528	0.07	
Error	32	65.5908	2.0497		
Corrected Total	53	166.8918			

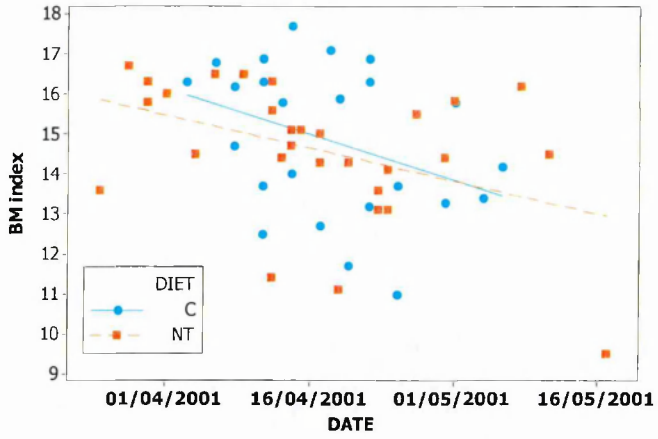


Figure 3. 4 Seasonal variation of the blastomere morphology (BM) index of Atlantic halibut in both dietary treatments. Control (C: blue circles) and nucleotide (NT: red squares).

Furthermore, a stepwise regression analysis revealed that apart from date, blastomere morphology was also affected by the condition of the females ($F = 2.69$, $p = 0.01$). Egg batches from females in a better condition –expressed by Fulton's condition index (K) (Eq. 2.6)- and spawned at the beginning of the season had the highest BM scores (Figure 3. 5). The equation was: $BM = 2477 - 0.0666\ Date + 0.219\ K$; with $p = 0.001$ and r^2-adj = 22.2.

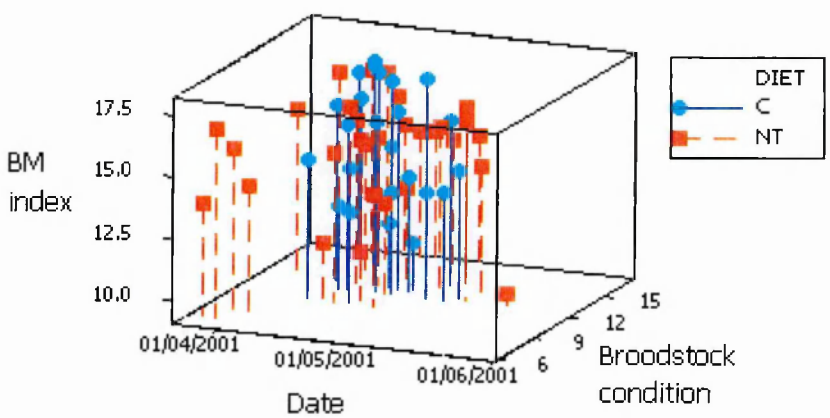


Figure 3. 5 Blastomere morphology (BM) index versus broodstock condition and date in Atlantic halibut eggs from control (C: blue circles) and nucleotide (NT: red squares) diets. There was no significant difference between diets.

Hatching rates

Microtitre plates were examined and the hatching rate recorded for each batch. Hatching rates were very low in both dietary treatments, ranging from 0 - 12 % in the control group compared

to 0 - 73 % in the nucleotide tanks. However, mean hatching rate was significantly higher in the nucleotide group (9.94 ± 4.22 % vs. 1.61 ± 0.95 %; W = 155.0, p = 0.0421) (Figure 3. 2). No significant differences were found between tanks (H = 5.35, p = 0.253) or between females (H = 15.94, p = 0.194) within dietary treatments. Furthermore, no trend was found over the season and hatching rate was also not correlated with blastomere morphology.

3.4.1.2 Egg physical parameters

Egg size, dry and wet weights and density were compared between both diets and the results are summarised in Table 3. 3.

Table 3. 3 Physical parameters of halibut eggs from the different diets along 2001season. Significant differences between diets are denoted by *. (Mean ± SEM).

Parameters	Control	Nucleotide
Wet weight (g)	0.0163 ± 0.0003	0.0165 ± 0.0005
Dry weight (mg)	1.4494 ± 0.0439	1.5802 ± 0.0447 *
Water content (%)	91.173 ± 0.108 *	90.298 ± 0.0736
Egg diameter (mm)	3.2911 ± 0.0430 *	3.2114 ± 0.0388
Density (g/ml)	0.8201 ± 0.0377	0.9556 ± 0.0481 *

Egg dry and wet weight

Halibut eggs from fish fed a nucleotide-enriched diet were significantly heavier than control ones in terms of dry weight (F = 4.35, p = 0.041; NT: 1.58 ± 0.04 mg vs. C: 1.45 ± 0.04 mg; Table 3. 3). A GLM showed that the dry weight of the eggs was significantly influenced by the diet and decreased over the season (Table 3. 4 and Figure 3. 6). There was also a tank effect since mean egg dry weight in one of the nucleotide tanks (F2) was significantly lower than that of tank R1 (also nucleotide).

Table 3. 4 General linear model analysis of egg dry weight of Atlantic halibut fed both dietary treatments. * Denotes significant effect. R^2-adj = 37.55 %

	DF	Sum of Squares	Mean Square	F Value	P Value
Diet	1	0.2410	0.2410	6.00	0.019*
Tank (Diet)	4	0.8277	0.2069	5.15	0.002*
Female (Diet Tank)	18	1.0265	0.0570	1.42	0.180
Date	1	0.3642	0.3642	9.07	0.005*
Error	37	1.4861	0.0402		
Corrected Total	61	3.9235			

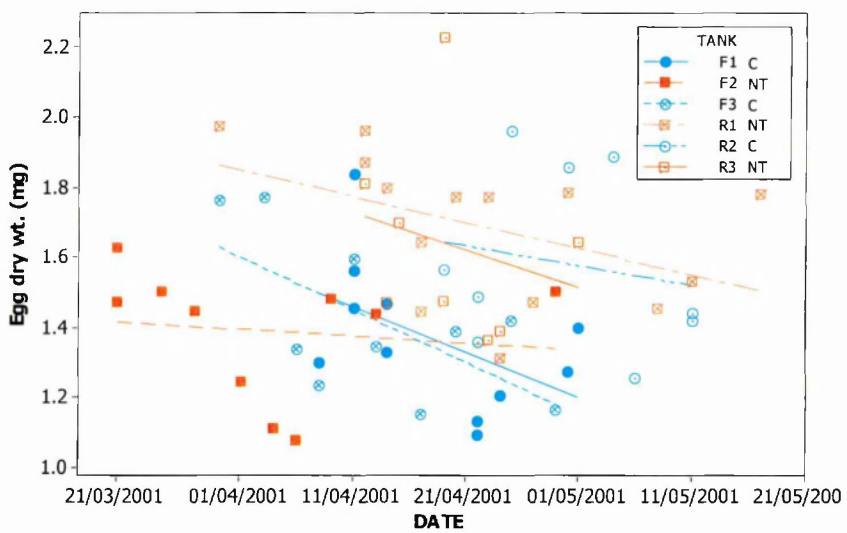


Figure 3. 6 Dry weight of Atlantic halibut eggs in the different tanks along the spawning season. Control tanks as blue circles and nucleotide as red squares.

However, the wet weight of halibut eggs was similar for both diets (C: 1.4494 ± 0.0439 mg; NT: 1.5802 ± 0.0447 mg; T = -0.28, p = 0.784; Table 3. 3). A GLM showed that the wet weight, as with dry weight, decreased along the spawning season (Table 3. 5; Figure 3. 7) and also that the eggs produced in the nucleotide tank F2 were significantly lighter than those from the other two nucleotide tanks (R1 and R3) (despite spawning biomass being similar between tanks: F = 0.60, p = 0.700). Both dry weight and wet weight were positively correlated with egg diameter (r = 0.211, p = 0.022 and r = 0.319, p = 0.016 respectively). Furthermore, water content was significantly lower in the eggs from the nucleotide tanks (F = 45.90, p = 0.000; Table 3. 3), with a concomitant increase in density for those eggs.

Table 3. 5 General linear model analysis of egg wet weight of Atlantic halibut fed both dietary treatments. * Denotes significant effect. R^2-adj = 36.32 %

	DF	Sum of Squares	Mean Square	F Value	P Value
Diet	1	$0.8 \cdot 10^{-6}$	$0.8 \cdot 10^{-6}$	0.22	0.644
Tank (Diet)	4	$0.72 \cdot 10^{-4}$	$0.18 \cdot 10^{-4}$	4.88	0.003*
Female (Diet Tank)	17	$0.97 \cdot 10^{-4}$	$0.57 \cdot 10^{-5}$	1.55	0.129
Date	1	$0.44 \cdot 10^{-4}$	$0.44 \cdot 10^{-4}$	12.04	0.001*
Error	38	$0.14 \cdot 10^{-3}$	$0.37 \cdot 10^{-5}$		
Corrected Total	61	$0.35 \cdot 10^{-3}$			

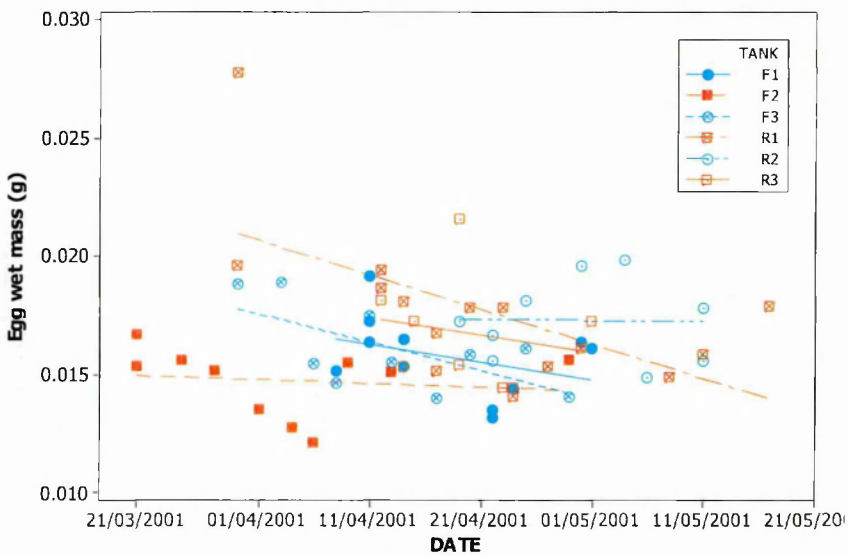


Figure 3. 7 Wet weight of Atlantic halibut eggs in the different tanks along the spawning season. Control tanks in blue circles and nucleotide ones in red squares.

Egg volume

The mean diameter of eggs from halibut fed nucleotide-enriched diets was 3.2114 ± 0.0388 mm, compared to 3.2911 ± 0.0430 mm of the control eggs. After doing a one-way ANOVA, differences between both diets appeared to be not significant (F = 1.90, p = 0.174). However, when a GLM was carried out, all the terms included in the model were significant (Table 3. 6). Control eggs were significantly bigger than nucleotides (F = 7.34, p = 0.011) and became smaller throughout the season (F = 7.15, p = 0.012), with some females performing better than others from the same tank (F = 3.26, p = 0.001). The tank effect within diet appeared to occur only in the control treatment.

Table 3. 6 General linear model analysis of egg diameter weight of Atlantic halibut fed both dietary treatments. * Denotes significant effect. R^2-adj = 52.05 %

	DF	Sum of Squares	Mean Square	F Value	P Value
Diet	1	0.1604	0.1604	7.34	0.011*
Tank (Diet)	4	0.2422	0.0606	2.77	0.046*
Female (Diet Tank)	18	1.2823	0.0712	3.26	0.002*
Date	1	0.1562	0.1562	7.15	0.012*
Error	29	0.6337	0.0218		
Corrected Total	53				

Egg density

The relative density, also called specific gravity, of the halibut eggs was also affected by the dietary treatment (Table 3. 3). In fact, those spawned by females fed the nucleotide-enhanced diet had a significantly higher density than the control ones (0.9556 ± 0.0481 $g \cdot cm^{-3}$ vs. 0.8201 ± 0.0377 $g \cdot cm^{-3}$; F = 4.96, p = 0.030). This parameter appeared not to change along the spawning season, since no linear or curvilinear regressions were significant. No tank effect was found either (F = 1.89, p = 0.113). Furthermore, egg density was negatively correlated with egg diameter (r = - 0.806, p < 0.0001).

3.4.2 Haddock Trial

3.4.2.1 Egg quality

Fertilisation rate and hatching rate were used as indicators to study the effects of the nucleotide supplementation on egg quality for the two consecutive spawning seasons that the haddock trial lasted.

Fertilisation rates

Fertilisation rate was higher in the NT-enriched diet in 2002 and 2003. However, differences were only significant in the first spawning season (χ^2 = 18.38, p = 0.000 and W = 5306.0, p =

0.1058 respectively); possibly as a result of the FR rate of the nucleotide group in 2003 being slightly lower than the previous year although not significantly (χ^2 = 1.17,p = 0.279; Figure 3. 8).

The pattern of variation in fertilisation rate throughout the spawning season differed between 2002 and 2003. In 2002, FR followed a dome-shaped distribution with batches from the middle of the spawning season having higher values compared to those from the beginning and end of the season (Figure 3. 9). Conversely, FR did not show a clear pattern in 2003, possibly as a consequence of the females from the same tank not spawning on similar dates but more spread throughout the season.

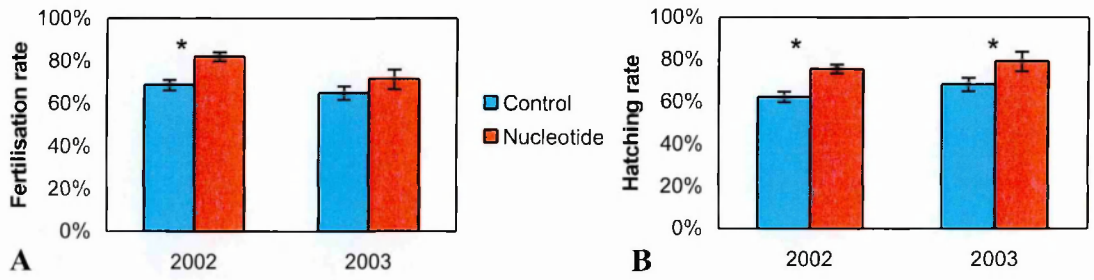


Figure 3. 8 Egg quality parameters for haddock. A) Fertilisation rate and B) Hatching rate. Significant difference is denoted by *. (Mean ± SEM)

Hatching rates

Mean hatching rates in the nucleotide treatment were 75.61 ± 3 % and 79.20 ± 3.24 % in 2002 and 2003 respectively and significantly higher (2002: W = 1311.5, p = 0.003; 2003: F = 4.09, p = 0.05) than the HR values for the controls in those years (62.33 ± 3.60 % and 68.37 ± 4.26 % respectively; Figure 3. 8).

Hatching rate also followed a dome-shaped pattern as FR throughout the first spawning season; in fact, both parameters were positively correlated in 2002 (r = 0.2843; p = 0.0106; Figure 3. 10). However, HR did not follow any characteristic trend along 2003 although it was highly correlated with FR (r = 0.5612; p = 0.0019).

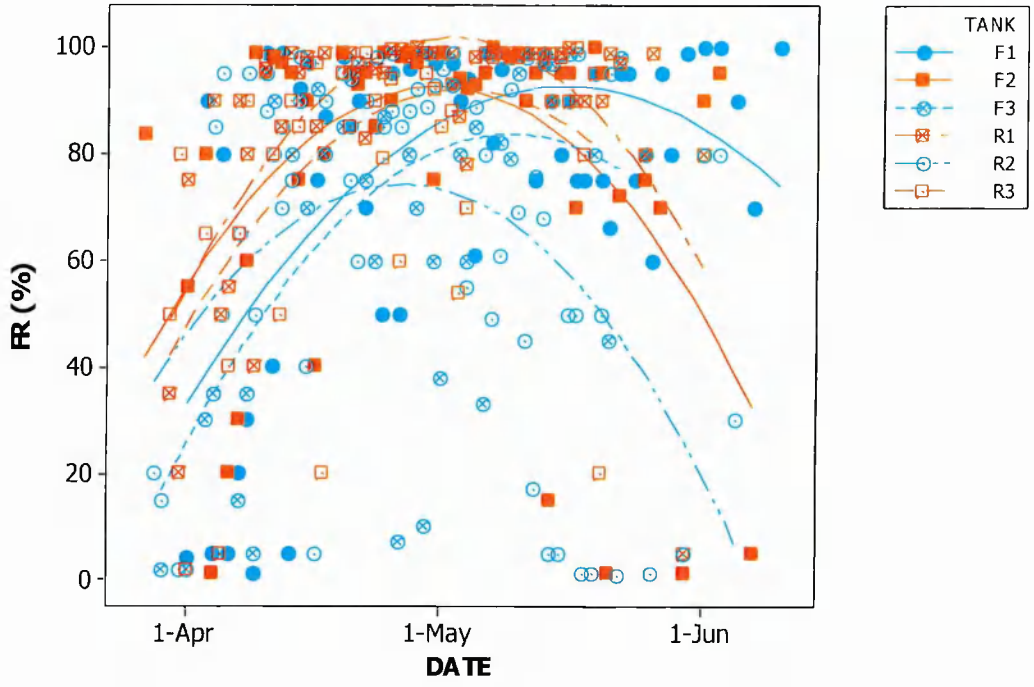


Figure 3. 9 Scatterplot of the fertilisation rate of haddock egg batches over the 2002 spawning season in the control (blue circles) and the nucleotide (red squares) tanks ($F = 69.29$, $p < 0.001$).

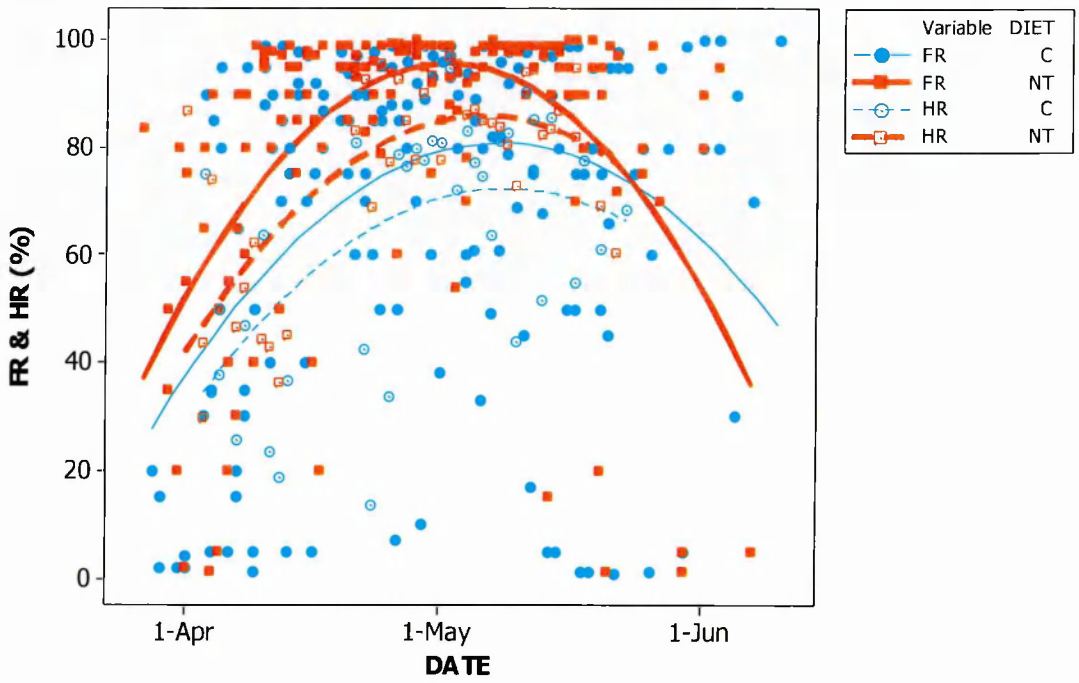


Figure 3. 10 Dome-shaped distribution of fertilisation rate (FR) and hatching rate (HR) of haddock egg batches over the 2002 spawning season. FR and HR were correlated (see text). Control (C: blue circles) and nucleotide (NT: red squares).

3.4.2.2 Egg physical parameters

Egg size, dry and wet weights and density were compared between both diets and the results are summarised in Table 3. 7.

Table 3. 7 Physical parameters of haddock eggs from the different diets over 2002 and 2003 seasons. Significant differences between diets are denoted by *. (Mean ± SEM).

	2002		2003	
Parameters	Control	Nucleotide	Control	Nucleotide
Wet weight (mg)	1.5577 ± 0.0128	1.5803 ± 0.012 *	1.6603 ± 0.0598	1.7558 ± 0.0463
Dry weight (mg)	0.1272 ± 0.0021	0.1253 ± 0.0020	0.1276 ± 0.0029	0.1521 ± 0.0034 *
Water content (%)	91.735 ± 0.171	91.741 ± 0.169	92.200 ± 0.262 *	91.155 ± 0.249
Egg diameter (mm)	1.4045 ± 0.0018	1.4108 ± 0.0019 *	1.4323 ± 0.0037	1.4433 ± 0.0051 *
Density (g/ml)	1.0527 ± 0.0096	1.0618 ± 0.0099	1.1404 ± 0.0427	1.1794 ± 0.0296

Egg dry and wet weight

The dry weight of haddock eggs was similar between control and nucleotide treatments in 2002 (C: 0.1272 ± 0.0021 mg; NT: 0.1253 ± 0.0020 mg; W = 31849.0, p = 0.5755). Data were then pooled and egg dry weight appeared to decrease over the first spawning season (r = - 0.2107; p = 0.0214; Figure 3. 11). No differences were found between tanks over the spawning season (p = 0.755).

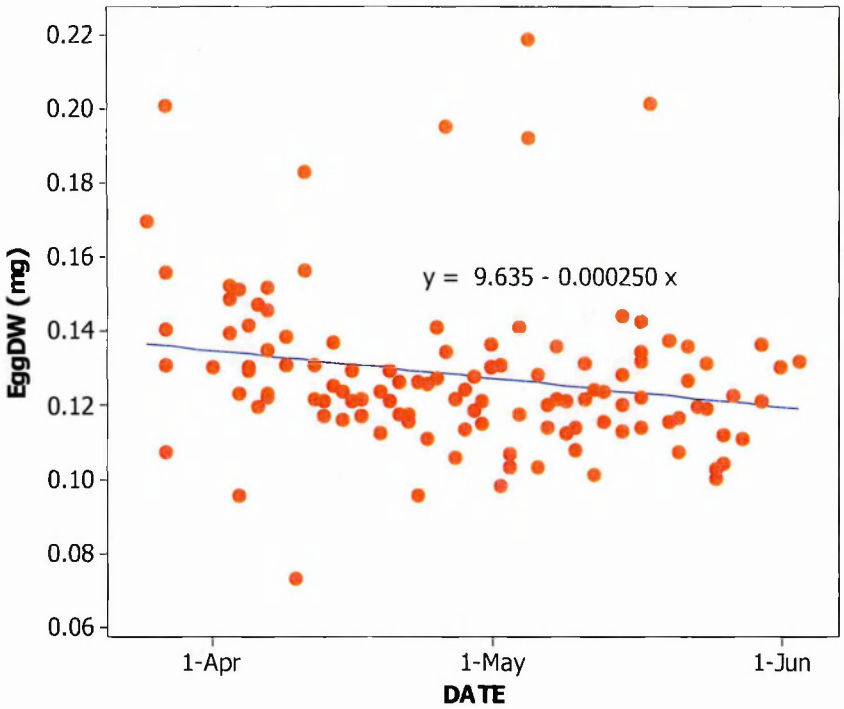


Figure 3. 11 Scatterplot of egg dry weight of haddock along the 2002 spawning season pooling the control and the nucleotide data.

However, in the second spawning season egg dry weight was significantly different between dietary treatments ($F = 20.91$; $p < 0.001$) in favour of the nucleotide group (0.1521 ± 0.0034 mg vs. 0.1276 ± 0.0029 mg; Table 3. 7). ANCOVA analysis was carried out using date as a covariate in order to study the seasonal variability over the second spawning season. Since the interaction "diet*date" was not significant ($F = 1.07$, $p = 0.307$), it was removed. The model was tested again without the interaction term and diet proved to be the only significant term in the model ($F = 19.70$, $p < 0.001$; Table 3. 8)

Table 3. 8 ANCOVA analysis of dry weight of haddock eggs from 2003 spawning season. * Denotes significant effect. Interaction Diet*date was not included, see text for explanation. R^2-adj = 30.06 %

	DF	Sums of Squares	Mean Square	F Value	P Value
Diet	1	0.0080	0.0080	19.70	0.000*
Date	1	0.0001	0.0001	0.32	0.551
Error	42	0.0171	0.0004		

No significant difference in wet weight was found between dietary treatments in 2002 (ANOVA: F = 1.64, p = 0.202; Table 3. 7). However, when GLM analysis was carried out using Diet and Tank as class variables and batch number as a covariate, the diet effect was significant (F = 4.09; p = 0.0439; Table 3. 9). A seasonal variability was also observed and eggs were heavier, on a wet-weight basis, at the beginning and end of the spawning season (Figure 3. 12).

Table 3. 9 GLM analysis of wet weight of haddock eggs from 2002 spawning season. * Denotes significant effect. R^2-adj = 21.41 %

	DF	Sum of Squares	Mean Square	F Value	P Value
Diet	1	0.1052	0.1052	4.80	0.029*
Tank (Diet)	4	0.5526	0.1381	6.30	0.000*
Date	1	1.2657	1.2657	57.76	0.000*
$Date^2$	1	1.2654	1.2654	57.75	0.000*
Error	351	7.8111	0.0223		

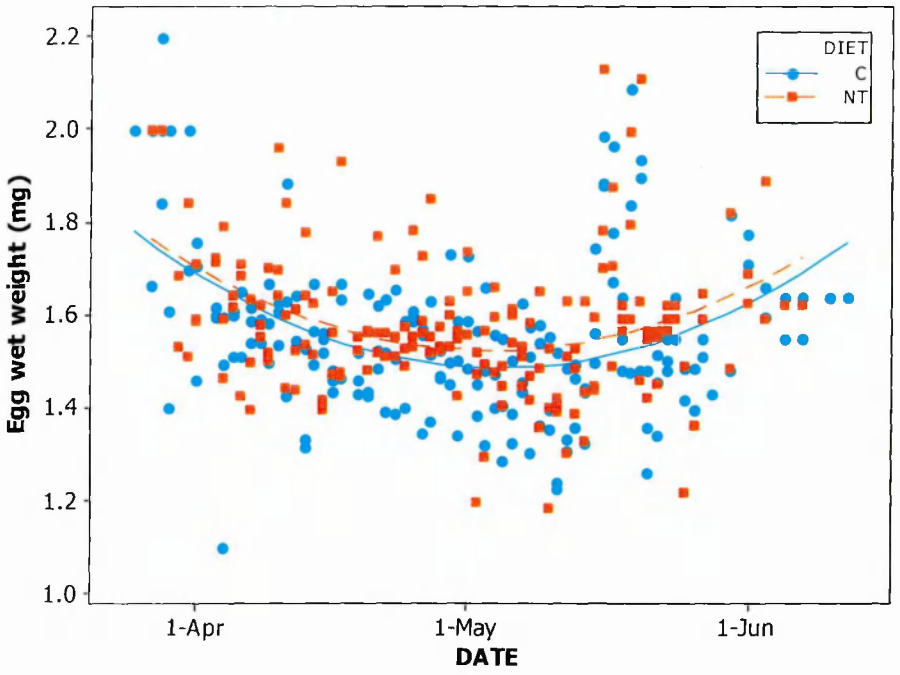


Figure 3. 12 Scatterplot of wet weight of haddock egg over the 2002 spawning season. Control (C: blue circles); Nucleotide (NT: red squares).

In 2003 however, the wet weight of haddock eggs was not significantly different between diets ($F = 2.54$, $p = 0.118$; Table 3. 7), and after GLM analysis neither tank nor seasonal effects were significant ($F = 1.71$, $p = 0.180$; $F = 0.19$, $p = 0.667$ respectively).

With regarding to water content of haddock eggs, differences between diets varied between 2002 and 2003 (Table 3. 7). Water content was similar in both diets in the first spawning season, averaging 91.735 ± 0.171 % and 91.741 ± 0.169 % for the control and nucleotide treatments respectively ($F = 0.50$; $p = 0.978$). However, water content of the eggs from the 'nucleotide-fed' females was significantly lower in 2003 (91.155 ± 0.249 %vs. 92.200 ± 0.262 %) compared to those from the control treatment ($F = 8.31$; $p = 0.006$).

Egg size

As explained in section 3.2.2.3, egg size was measured as the egg diameter for all the batches collected. Egg diameter changed during the season, and larger eggs were released in the first batches and their size reduced as the season progressed (Figure 3. 13). Mean egg diameter was significantly higher for the nucleotide diet in 2002 (1.4108 ± 0.0019 mm vs 1.4045 ± 0.0018 mm; $p = 0.026$) and 2003 (1.4433 ± 0.0051 mm vs. 1.4323 ± 0.0037mm; $p = 0.0444$) (Table 3. 7).

GLM analyses were carried out in order study the variation in egg diameter in 2002 and 2003 using diet and tank as class variables and batch number (BN) as a covariate (Table 3. 10). There was a diet and BN effect in both years (2002: $F = 9.30$, $p = 0.0023$; $F = 631.34$, $p < 0.0001$; 2003: $F = 14.62$, $p < 0.0001$; $F = 201.80$, $p < 0.0001$; diet and BN respectively). The interaction "diet*BN" was also significant in both years (2002: $F = 8.09$, $p = 0.0045$; 2003: $F = 17.43$, $p < 0.0001$) and was studied separately each year using least mean squared analysis. It appeared that in 2002 the first 24 batches of nucleotide eggs were significantly bigger than control eggs, thereafter size remained similar between both diets until the 54th batch when control eggs were significantly bigger than nucleotide ones (Figure 3. 13). A similar variation was also observed during the following spawning season, but in this case nucleotide eggs were bigger until the 12th

batch and smaller than those from the control females from the 23rd batch onwards (Figure 3. 13).

Table 3. 10 GLM analyses of the egg size of haddock in 2002 and 2003 spawning season. * denotes significant effect. 2002: R^2-adj = 39.61 %; 2003: R^2-adj = 31.88 %.

	Parameter	DF	Sum of Squares	Mean Square	F Value	P Value
2002	Diet	1	0.0173	0.0173	9.30	0.002*
	Tank (Diet)	4	0.6952	0.1738	93.66	0.000*
	BN[1]	1	1.1715	1.1715	631.34	0.000*
	Diet*BN[1]	1	0.0150	0.0150	8.09	0.005*
	Error	1749	3.2453	0.0019		
2003	Diet	1	0.0483	0.0483	14.62	0.000*
	Tank (Diet)	3	0.0073	0.0024	0.74	0.531
	BN[1]	1	0.6666	0.6666	201.80	0.000*
	Diet*BN[1]	1	0.0576	0.0576	17.43	0.000*
	Error	536	1.7705	0.0033		

[1]: Batch number

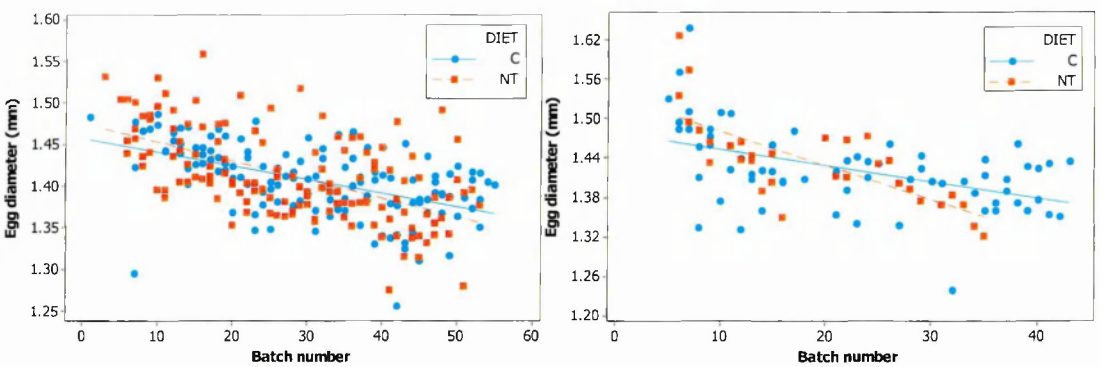


Figure 3. 13 Variation of egg size of haddock broodstock over 2002 (left) and 2003 (right) spawning seasons. Control (C: blue circles); Nucleotide (NT: red squares).

Egg diameter was positively correlated to egg dry weight in 2002 and 2003 (2002: r = 0.2816, p = 0.0047; 2003: r = 0.6630, p < 0.0001) and also to wet weight (r = 0.4727, p < 0.0001 and r = 0.6467 p < 0.0001 respectively) in both spawning seasons (Figure 3. 14).

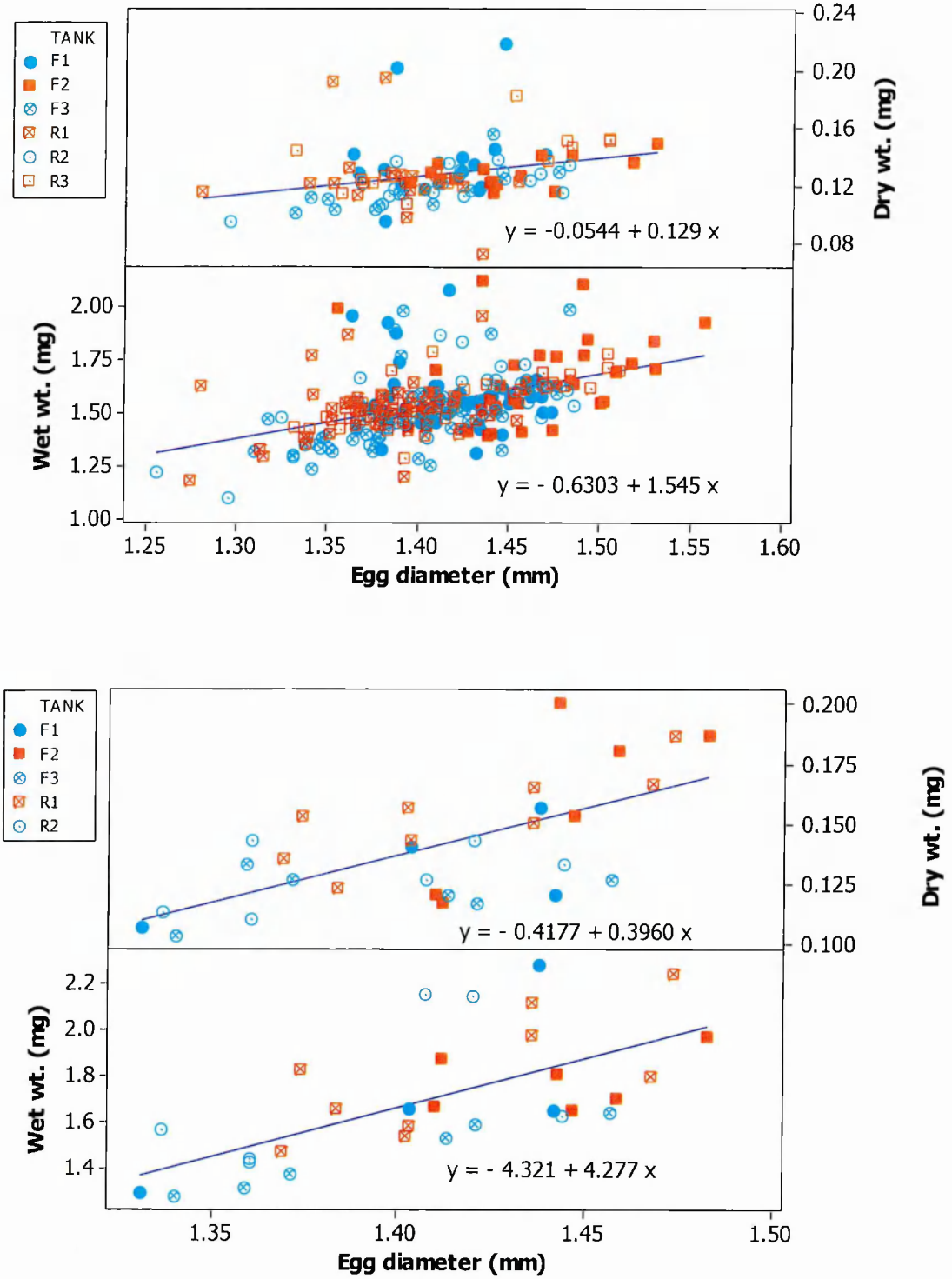


Figure 3. 14 Scatterplots of dry and wet weights against diameters of haddock eggs from both dietary treatments in 2002 (top graph) and 2003 (bottom graph). Control: Blue circles and Nucleotide: Red squares.

Egg density

Specific gravity of haddock eggs was evaluated along the 2002 and 2003 spawning seasons for each diet. Mean values of egg density were 1.0527 ± 0.0096 g cm^{-3} and 1.0618 ± 0.0099 g cm^{-3}

(control and nucleotide respectively) with differences being non-significant ($p = 0.2229$) between both diets in 2002 (Table 3. 7). In the same year, egg density was positively correlated with date ($r = 0.4681$, $p < 0.0001$) and BN ($r = 0.4796$, $p < 0.0001$), indicating that density increased as the season progressed (Figure 3. 15), probably as a result of egg volume decreasing throughout the season. Therefore egg density decreased with egg size (Figure 3. 15).

During the following year, mean egg density was almost significantly different between both diets ($W = 286$, $p = 0.0721$), suggesting that there might have been a trend and specific gravity of the nucleotide eggs was greater than in the control group (Table 3. 7). Variation along the second spawning season was not significantly correlated with any seasonal parameter (date, temperature and BN) when using either pooled data or data separated by diet.

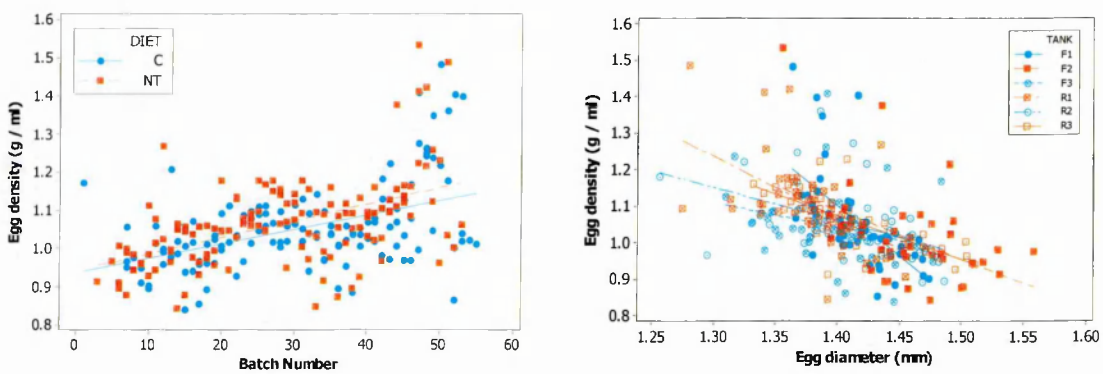


Figure 3. 15 Variation of the density of haddock eggs over the spawning season (left) and with egg diameter (right) in 2002. Control (C: blue circles); Nucleotide (NT: red squares).

3.5 Discussion

Up until now, the effects of nucleotide supplementation of broodstock diets on egg quality and physical characteristics had never been studied. The potential benefits of nucleotide-enriched diets on them will be discussed in this section.

3.5.1 Effects on egg quality

Potential effects of nucleotide supplementation of the broodstock diets on egg quality were assessed by comparing the fertilisation and hatching rates of halibut and haddock eggs. Additionally, a blastomere morphology index was also studied in the eggs collected from the halibut broodstock.

Mean fertilisation rates (FR) and blastomere morphology values of halibut eggs were similar between both dietary treatments (Figure 3. 2). Fertilisation rate showed a dome-shape distribution with batches spawned at the middle of the season having higher fertilisation success compared to those at the beginning and end of the season. The rate of decrease of FR at the end of the season was slower in the nucleotide group compared to the control treatment (Figure 3. 3) and this could be indicative of a relatively higher quality of the nucleotide eggs at the end of the spawning season, when female resources are at their lowest. Mean fertilisation rates and blastomere morphology scores were much higher and similar respectively to the FR and BM reported by Mazorra *et al.* (2003) also for Atlantic halibut. The latter parameter decreased over the season indicating that cleavage abnormalities increased with the season although unlike studies by Kjørsvik *et al.* (2003) using turbot eggs, no direct correlations were found between blastomere morphology and fertilisation rate nor with hatching rate. Results of the present study have shown that blastomere morphology varies not only along the season, but also depends on

broodstock condition (Figure 3. 5); this had never been reported up until now. Thus, broodfish in a worse condition at the end of the spawning season, and probably also at the beginning of the season, produced egg batches with more abnormalities. In contrast, the mean hatching rate of eggs from the nucleotide group was significantly higher than with the control diet, although no seasonal trend was found. However, hatching rates of halibut eggs in our study (Figure 3. 2) were much lower than those reported in other studies (28-51%) and under the same environmental conditions (Mazorra *et al.* 2003). Determination of the ovulatory rhythms is very important in Atlantic halibut in order to avoid under-, overripening and maximise egg viability (Norberg *et al.*, 1991). Overripening of eggs after ovulation seems to occur during a shorter time period for batch spawners than for fish spawning once a year (Kjørsvik *et al.*, 1990). Stripping time in relation to ovulation has proved to be crucial not only for egg yield but for high fertilisation and hatching rates in halibut (e.g. Norberg *et al.*, 1991; Bromage *et al.*, 1994) and other batch spawner species such as turbot (McEvoy, 1984; Howell & Scott, 1989). However even by considering the ovulatory rhythms for individual fish, as in this study (§ 2.3.2.1.1), fertilisation rates and egg viability are highly variable in egg batches stripped from halibut (Kjørsvik & Homelfjord, 1995). The cause of these low hatching rates in our study was unknown but hatching rates of different batches with high fertilisation rates can vary greatly in halibut. Nevertheless, in the unlikely event of having used inadequate incubating conditions the higher hatching success of the nucleotide eggs may indicate a better embryo resistance under adverse conditions. Since there were no differences in the milt motility between both treatments (§ 2.3.2.1) this significant improvement of the hatching rate may be a result of the better quality of the nucleotide eggs themselves, perhaps due to differences in their composition (see below and Chapter 5).

With regarding to haddock, fertilisation and hatching rates were higher in the nucleotide treatment in both spawning seasons. Our results for fertilisation rate are much higher than those obtained using mated pair haddock (FR = 38.5 %) and that appeared to be associated with low spermatocrit and thickening of milt over the season (Trippel *et al.*, 1998). Environmental conditions may affect egg development and hatching (Kjørsvik *et al.*, 1990; Bromage, 1995;

Brooks *et al.*, 1997). However, incubating conditions in the control and nucleotide treatments were the same in our study, so differences in hatching as a result of a differing environment can be excluded. The seasonal pattern of fertilisation and hatching rates differed between 2002 and 2003. In the former, fertilisation and hatching success followed a dome-shape distribution, both parameters being highly correlated, however in the latter no seasonal variation was observed despite fertilisation and hatching rates being also correlated.

Blastomere morphology could not be assessed in the haddock trial since eggs were at the morula-stage, having passed the 8 – 32 cell stage (Shields *et al.*, 1997; Vallin & Nissling, 1998; Kjørsvik *et al.*, 2003; Rideout *et al.*, 2004), when collected daily. Blastomere morphology, also described as early cleavage patterns, have been confirmed recently as a reliable 'tool' to predict the embryo viability also in haddock as hatching rates are inversely correlated with the percentage of abnormal eggs in terms of symmetry, margins, cell size and adhesion (Rideout *et al.*, 2004). Based on this, it might be inferred from our results that as the hatching rates of haddock eggs were significantly different between diets, the nucleotide eggs had a lower percentage of abnormalities when compared to those from the control group.

The underlying cause of the improvement of haddock egg quality is unknown. As explained in chapter 2, since gadoid fish can die as a result of stress if manipulated during spawning (Cutts & Shields, 2001) haddock were not stripped and fertilised eggs collected, therefore it was not possible to assess the milt motility in this species. Broodstock nutrition can affect milt characteristics and also fertilisation (Dabrowski & Ciereszko, 1996; Asturiano *et al.*, 2001). Nevertheless, assuming that as in halibut, the nucleotide diet did not affect the motility of the haddock milt, the improvements observed in the fertilisation rate and hatching rate of the eggs from the nucleotide tanks will be a consequence of higher quality eggs. It could then be hypothesised that the composition of the eggs is different depending on the diet fed to the broodstock. This issue will be addressed in chapter 5, where potential differences in egg composition with regard to nucleotide levels will be discussed.

3.5.2 Effects on egg physical characteristics

The effects of nucleotide supplementation on egg physical characteristics of the two species were studied by comparing the size, weight, water content and density of the collected eggs from nucleotide and control broodstock and assessing their variation throughout each spawning season.

Halibut egg size was significantly different between both dietary treatments with eggs from nucleotide females smaller than those from the control group. Nevertheless, the differences between both groups were very small and mean egg diameters fall within the range of 3.06 - 3.49 mm reported by Haug (1990) for Atlantic halibut. Kjesbu *et al.* (1992) gave the first evidence that egg diameter is positively correlated with the broodfish weight, based on studies of individual females over two consecutive spawning seasons. However, this can be excluded as a reason for nucleotide eggs being smaller than controls since no significant difference in the weight spawning females was observed between diets (C: 8.44 ± 0.47 kg; NT: 8.00 ± 0.78 kg; $F = 1.63$, $p = 0.214$). Variation in halibut egg size was studied at an individual level and in most cases followed the expected decreasing pattern along the season, however this trend was not confirmed in some females; probably as a consequence that some egg batches were missed and could not be sampled because the female 'dumped' it into the tank water before stripping was carried out.

Conversely, the average size of haddock eggs from the nucleotide treatment was significantly larger than in the control group during 2002 and 2003. The egg diameters observed were similar to those reported in previous experiments with haddock (Hislop *et al.*, 1978; Hislop, 1984). The increase in size from the first to the second spawning season (from 2 to 3 years of age) (Table 3. 7) was also described by Hislop (1988) in eggs of haddock of the same age as in the present study. This phenomenon is also observed in other important gadoids such cod (Kjesbu *et al.*,

1992) and suggests that egg size takes priority over the actual fecundity of the fish (Kjesbu *et al.*, 1996). Haddock may therefore also follow the same reproductive strategy.

Seasonal variation could not be studied at individual level in haddock since the parental origin of the batches of eggs could not be determined. Haddock eggs from the nucleotide treatment were significantly larger at the beginning of the spawning season, became of similar size towards the middle and smaller than control eggs at the end of the spawning season. Nucleotide eggs decreased in size more steeply than those from the control group and this pattern was observed along the two consecutive spawning seasons. Egg size decreased over the season in both halibut and haddock. This phenomenon of decreasing in size has been described in previous studies with Atlantic halibut (Kjørsvik *et al.*, 1987) and haddock (Hislop *et al.*, 1978). Other batch spawner species such as cod, whiting, Norway Pout, turbot, yellowtail flounder and sole are known to reduce the size of the eggs produced throughout the spawning season (e.g. Hislop, 1984; Houghton *et al.*, 1985; Bromley *et al.*, 1986; Manning & Crim, 1998) and it has been associated with a reduction of the resources in the female (Hsiao *et al.*, 1994), with the rate of decrease in egg size being dependent on the nutritional status of the fish (Kjesbu *et al.*, 1991).

As explained by Blaxter (1969), egg size is inversely related to fecundity and fish producing bigger eggs tend to release a lower number of eggs (i.e. lower fecundity). In our study, the size of the eggs produced by halibut females fed the nucleotide diet was significantly smaller than those produced by the control group (Table 3. 3). Therefore, this might help to explain the greater fecundity of the halibut females fed the nucleotide-enriched diet (Table 2. 3). The increase in the size of haddock eggs between 2002 and 2003 may also explain the decrease in fecundity between both spawning seasons confirming that in haddock as in cod, egg size takes priority over fecundity (Kjesbu *et al.*, 1996).

Regarding the other parameters, similar wet weights were recorded in halibut eggs from both treatments, although the mean dry weight of the eggs from the nucleotide group was significantly higher when compared to those from the control tanks, indicating a higher content

of water in the control eggs (Table 3. 3). Water content of halibut and haddock eggs was similar to that reported by Craik & Harvey (1987). The difference in dry weight between both dietary treatments might indicate a higher incorporation of maternal resources into the oocytes during oogenesis in the halibut females fed the nucleotide enhanced diet.

Water content and dry weight of haddock eggs were similar between both diets in 2002, despite the fact that the wet weight of eggs collected from the nucleotide tanks was significantly higher. However, in the second year, the water content was significantly higher and the dry weight significantly lower in the control when compared to the nucleotide eggs (Table 3. 7). Therefore, as in halibut, it appears that larger amounts of non-aqueous compounds were transferred into the eggs of haddock fed the nucleotide-enhanced diet during the second spawning season.

No clear seasonal pattern over the spawning season was observed in the density of haddock eggs in 2003. However, density of halibut and haddock eggs – in 2001 and 2002 respectively– increased, and hence buoyancy decreased, as the egg became smaller. Similar results have also been obtained in other teleosts species such as cod (Kjesbu *et al.*, 1992; Nissling *et al.*, 1994) and sprat (Nissling *et al.*, 2003). From the present results it can be inferred that the buoyancy of eggs from the later batches was probably lower than those from earlier ones.

Halibut eggs from the nucleotide treatment were denser than in the control and this was related to a lower water content in the nucleotide eggs. Since high water content, rather than lipid, is the principal cause of buoyancy in the eggs of marine species (Craik & Harvey, 1987) and the water content of haddock nucleotide eggs collected in 2003 was lower than in the control eggs from the same year, it is likely that the assumption that nucleotide haddock eggs being significantly more dense than the control eggs was correct. However, although increments of the dry weight of the nucleotide eggs were associated with its higher density, it may seem contradictory that halibut eggs from the nucleotide group decreased in size while haddock ones became larger when broodstock were fed a nucleotide-enhanced diet. This phenomenon might be therefore a reflection of the different biology and reproductive strategies of both species. In halibut the

nucleotide supplementation of the diet produced an increase of the fecundity and therefore, since egg size and fecundity are inversely related parameters (Blaxter, 1969), egg size was reduced. Whereas haddock fed the nucleotide diet, in which improved fecundity was not clear, the size of the egg was increased, suggesting that as in other gadoids, such as cod, egg size takes priority over the actual fecundity of the fish (Kjesbu *et al.*, 1996).

Implications of differences in the specific gravity of the eggs are not relevant for the development of the eggs in our study because upwelling incubators in which the eggs were moving continuously in the water column of the tank were used to maintain them until hatch. However in the wild alterations of the density can be critical for the survivorship of the developing embryos (Nissling *et al.*, 1994; 2003).

Chapter 4: Larval quality & performance

4.1 Introduction to larval quality

As with eggs, larval quality is of critical importance both in the wild and in cultivation systems. There is no point in having highly viable eggs with high survival rates if the subsequent larvae present abnormalities or deformities that might compromise their development to juveniles or even cause death. According to Kjørsvik & Homelfjord (1995), larval quality should be estimated by the end of the period of endogenous feeding, at the end of the yolk-sac stage. The term larval quality has been widely used in the scientific literature, particularly in ecological and nutritional studies involving fish, however, there is not a clear definition. Instead of larval quality, however, larval *functionality* may be a more suitable assessment of larvae and should be estimated by morphological development, pigmentation pattern, survival rate, resistance to stress or by response to a stimulus. Larval functionality depends, to a large extent, on the developmental history and environmental conditions during both egg and larval development (Kjørsvik & Homelfjord, 1995). The main indicators of larval quality and factors influencing it are discussed in the present section.

4.1.1 Factors affecting larval quality

As explained in chapter 3, there are many factors that may affect egg quality. Therefore, since good eggs generally produce larvae of good quality, many factors also influence the quality of the larvae (Fig 3. 1).

There are many studies reporting that environmental conditions for egg and larval incubation affect greatly larval viability. Optimal temperatures for egg and larval development depend on the species. However, rearing at the extremes of their temperature range will often provoke the

development of abnormalities and even the death of the larvae before becoming juveniles (e.g. Ørnsrud *et al.*, 2004). In addition to the direct effects of temperature on the larvae, water temperature in the broodstock tanks can affect the quality of the eggs and therefore larval 'functionality'. Salinity is also an important factor during the larval stage (Holliday, 1969). The ability of the larvae to survive changes of salinity will depend on their osmoregulatory ability and the energy required for osmoregulation influences the efficiency of yolk utilisation and growth rate of the larvae. The salinity of the water affects not only the buoyancy of eggs but also of larvae. Larvae that need to gulp air to fill the swim bladder might not be sufficiently buoyant to reach the surface if salinity is too low. In summary, suboptimal salinities affect survival and growth of the larvae (e. g. Tandler *et al.*, 1995a). Light is also important during larval rearing and spectrum, intensity and photoperiod, all influence the performance of developing larvae (e. g. Downing & Litvak, 2001; 2002; Downing, 2002).

The quality of the water also affects the viability of the larvae. The mere metabolic activity of the larvae themselves deteriorates water quality and in addition the food and waste organic material may cause alterations of pH, concentrations of dissolved oxygen, carbon dioxide and nitrogenous compounds. The importance of maintaining larvae in cultures with a continuous supply of high quality water is therefore critical to assure larval viability.

Broodstock and larval nutrition also both have a major impact on larval quality. The importance of broodstock nutrition on larval quality was discussed in section 2.1.1; suboptimal feeding of broodfish may increase body deformities and swim bladder non-inflation in larvae (e. g. Cerda *et al.*, 1994a; Tandler *et al.*, 1995b). The importance of larval nutrition on survival and viability of fish larvae is highlighted by the great number of nutritional studies carried out to discover the nutritional requirements of larvae in different cultured species. The role of nutritional components affecting skeletal development, and therefore deformities, was reviewed by Cahu *et al.* (2003). For instance, dietary incorporation of phospholipids in the diets reduces the incidence of spinal malformations; tryptophan deficiency in the diet induces scoliosis in salmonids and high levels of vitamin A have teratogenic effects by increasing the incidence of

bone deformities. Evidence of swim bladder stress syndrome (SBSS) in cod larvae linked with larval nutrition was given by Shields *et al.* (2003).

Stress is unavoidable in fish farming, although it can be minimised. Larval fish should be given the same consideration as adult fish when attempting to minimise stress under aquaculture conditions (reviewed by Pickering, 1998). Suboptimal environmental conditions, water quality, nutrition, handling and husbandry practises will all put larvae under stress. The impact of stress on the performance of the fish larvae can be observed in changes of behaviour, depleted growth and increased mortality rates. Stressed fish are more susceptible to diseases and fungal, bacterial and viral infections tend to increase. In addition, many aspects of the defence systems of the animal, both non-specific (mucus layer, phagocytic cells, complement, etc.) and specific systems (lymphocytes, immunoglobulins, etc), are known to be inhibited by stress (e. g. Manning, 1998; Pickering, 1998). Furthermore, studies on cod have showed that larval quality is affected when broodstock are stressed. Stressed cod broodfish can reproduce displaying fewer courtships and altered courtship sequences but the amount of abnormal larvae was much higher than in non-stressed fish (Morgan *et al.*, 1999).

4.1.2 Larval Quality indicators

Egg quality, resistance to stress, deformities (opercular, spinal – lordosis (dorsal deformity, ∨ shape); kyphosis (ventral deformity, ∧ shape) and scoliosis (lateral deformity, zig-zag shape)–, mandibular, fins, lateral line), swimbladder development, pigmentation, starvation, behaviour, morphometric and biochemical analyses have all been used in some way to report the quality of the larvae.

Ideally, as with eggs, these predictors should not require lengthy or sophisticated lab procedures and should be carried out early during larval development in order to avoid using unnecessary holding facilities and staff time.

Resistance to stress

Stress tests are based on the assumption that the weaker larvae are less tolerant to the treatment and will die more rapidly than healthier and stronger larvae. As expressed by Lavens *et al.* (1995), it was Japanese scientists who first evaluated larval viability using simple procedures that consisted of exposing them to stressors (i.e. lifting the larvae from the water and exposing them to air) and measuring their recovery and survival afterwards. A stress test has also been used to evaluate the quality of fish fry by exposing the larvae to water of high salinities (55 – 65 ‰) and measuring survival at regular time intervals (Dhert *et al.*, 1992). However, as Howell *et al.* (1998) suggested, short-term 'stress tests' involving different temperature and salinities may lack sensitivity to detect some important physiological differences and should therefore be interpreted with caution.

Other types of stress test are those so-called, starvation tolerance tests also carried out to check the quality of the larvae. Blaxter & Hempel (1963) described the point of no-return (PNR) as the time at when the cumulative effects of starvation become irreversible and 50% of starved larvae are still alive but are unable to feed even when food resources become available. Another parameter used in the survival activity index (SAI) which can be calculated from the number of surviving larvae and the survival duration. This index has been used successfully in a wide variety of species such as scorpaenoid fish - *Sebastiscus marmarotus* - (Shimma & Tsujigado, 1981), striped jack - *Pseudocaranx dentex* - (Watanabe *et al.*, 1998), Japanese flounder – *Paralichthys olivaceus* - (Furuita *et al.*, 2000, 2002, 2003b), and mangrove red snapper – *Lutjanus argentimaculatus* - (Emata & Borlongan, 2003).

Egg quality

Egg quality parameters (e.g. cell morphology, fertilisation rate, hatching rate) are highly related to larval viability and *functionality*, and this phenomenon has been observed in many species. Cell morphology has been demonstrated to be a predictor of larval viability of cod (*Gadus morhua*) (Kjørsvik, 1994). Thus, when cod eggs were separated into groups characterised as

'normal' and 'abnormal' blastulae, larvae hatching from the 'abnormal' groups were less pigmented and active than those from 'normal' eggs. In turbot, *Scophthalmus maximus*, females with the highest fertilisation and hatching rates presented not only the highest larval survival, but also the largest number of normally metamorphosed larvae (Kjørsvik *et al.*, 2003). Furthermore in the same study, fertilisation rate and blastomere morphology were positively correlated to hatching rate, percentage of survival, normal metamorphosis and normal pigmentation of turbot larvae. The issue over egg size as a predictor of larval quality might, as with egg quality, be a subject for a long discussion since while some studies report larval size, growth and survival during the yolk-sac stage to correlate significantly with egg size (e.g. Nissling *et al.*, 1998), others do not.

Swim bladder

During early larval development, many physoclistous species (i.e. without any connection between the swim bladder and oesophagus) pass through a brief period when a rudimentary pneumatic duct connects the foregut to the swim bladder. During this period it is believed that air gulping is indispensable for the initial inflation of the swim bladder, requiring larval interaction with the water surface. However, accumulation of films on the water surface disturbs inflation (Chatain & Ounais-Guschemann, 1990) and the use of surface skimmers, consisting of a blower and a floating trap, are now widely used during larval rearing. Environmental factors such as light intensity, tank colour, photoperiod, temperature and turbidity among others are known to affect the initial ability of larvae to inflate their swim bladders (e. g. Martin-Robichaud & Peterson, 1998). Tandler *et al.* (1995b) also reported that apart from larval growth and survival, swim bladder inflation is also directly affected by the composition of the broodstock diet. Failure to carry out the first inflation results in a lack of correct buoyancy control and swim bladder atropy, this being associated with skeletal deformities (lordosis, scoliosis) in numerous cultured species such as seabass, striped bass, red seabream and gilthead seabream (e. g. Martin-Robichaud & Peterson, 1998; Planas & Cunha, 1999). Larval deformities will be discussed in the next section.

Conversely, swim bladder hyperinflation, provokes the so-called 'swim bladder stress syndrome' (SBSS): larvae float near the water surface and stop feeding and swimming, dying in a few days due to starvation. Different investigations have suggested that sub-optimal environmental conditions (temperature, salinity, water depth, N_2 levels) may induce stress related swim bladder abnormalities in larval fish (Johnson & Katavic, 1984; Bagarinao & Kungvankij, 1986; Planas & Cunha, 1999). However, more recently Shields *et al.* (2003) gave the first evidence that swim bladder overinflation is also influenced by the nutritional status of larval fish reared under uniform environmental conditions.

Skeletal abnormalities

A wide variety of skeletal abnormalities such as those affecting the spinal column, fins, operculum, and jaws, have been described in cultured fish.

Spinal deformities in larvae are common and include lordosis, kyphosis and scoliosis. As mentioned above, lordosis and scoliosis are associated with the absence of a normal swim bladder. Larvae with malformed swim bladders have difficulty maintaining their position in the water column and have to swim at oblique angles with rapid fin strokes. This induces distortions of the spinal column in order to compensate for the oblique direction of the body axis (Watanabe & Kiron, 1995). Nutritional deficiencies such as low n –3 HUFA content have also been associated with this phenomenon in red seabream larvae: the larvae presented low swimming activities, did not swim up to the surface to inflate the swim bladder and also developed spinal deformities (Watanabe & Kiron, 1995). This and other nutritional components affecting skeletal development of fish larvae were recently reviewed by Cahu *et al.* (2003).

The operculum – the bony flap that covers the gills – is comprised of four bones: opercle, preopercle, interopercle and subopercle. These bones may also suffer abnormalities and such deformities are associated with severe foldings and twists of the operculum, affecting the morphology and biological performance of the fish (Koumoundouros *et al.*, 1997; Planas & Cunha, 1999). Causative factors such as nutritional deficiency (e.g. vitamin C), heritability and

environmental factors have been discussed (e.g. Planas *et al,* 1999) although are yet unclear. Electron microscopy observations on 30-day-old seabream larvae showed abnormal mineral deposition within the dermal ossification in curled opercles, suggesting that an early alteration in the connective tissue formation and an abnormal mineralisation of the fibrous bone can predispose opercular folding (Galeotti *et al.*, 2000).

Other abnormalities such as jaw malformations and missing or additional fin rays have also been reported in larvae of most cultured species such as Dover sole, halibut, Japanese flounder, red seabream, gilthead seabream and seabass.

The occurrence of all these conditions has been observed both in wild and cultured larvae. However, as reported by different studies, their incidence is usually higher in reared fish (gilthead seabream: Boglione *et al.* (2001); red seabream: Matsuoka (2003)). This fact, suggests a quality gap between wild-caught and hatchery-reared specimens pointing out the existence of improvement margins in rearing techniques.

A wide variety of factors are implicated in inducing abnormalities in fish larvae, and in general the causes are poorly understood. The contribution of genetic-related disorders such as mutation and inbreeding should not be discounted (e. g. Afonso *et al.*, 2000). Sub-optimal nutritional and environmental factors are also known to affect abnormalities in fish larvae.

Pigmentation

Flatfish often exhibit abnormal pigmentation under culture conditions, especially of the ocular side. Studies in different flatfish species have confirmed that the abnormal pigmentation may be a result of inadequate nutrition during the larval stage (e. g. Bell *et al.,* 2003). The occurrence of malpigmented juveniles can be reduced through the supplementation of prey enriched with fatty acids, phospholipids and vitamin A. A positive correlation was found between pigmentation success and the ratios of DHA/EPA in the total and polar lipid fractions of turbot larvae so the amounts of these PUFA in the phosphatidylethanolamine fraction of the larvae were suggested

to be important for pigmentation (Rainuzzo *et al.*, 1994). Estévez *et al.* (1999) and McEvoy *et al.* (1999) showed negative effects of increasing levels of dietary arachidonic acid on pigmentation success of turbot and halibut respectively. Furthermore, Estévez *et al.* (2001) induced a higher incidence of malpigmentation in Japanese flounder larval fed enrichments with an EPA/ARA ratio of 0.3 compared to those fed an EPA/ARA ratio of 4.1. The importance of lipid nutrition has been recently reviewed by Bell *et al.* (2003). A deficiency in dietary vitamin A, which affects the process of vision and neural transmission, also resulted in poorly pigmented halibut larvae (Ronnestad *et al.*, 1998).

Morphometric parameters

Measurements of the larval body can also provide information on condition. Some body measures are considered to be sensitive to food deprivation (e.g. body height above the pectoral fin and above the anus, myotome height, inter-orbital distance, dry weight), while other measures, such as total length, eye diameter and head length, are considered relatively insensitive to starvation (Ehrlich *et al.*, 1976; Theilacker, 1978). Morphometric indices resulting from dividing starvation-sensitive parameters by 'non-starvation-sensitive' parameters have been widely used to assess the condition of larval fish (e. g. Koslow *et al.*, 1985; Ferron & Leggett, 1994). For instance, since shrinkage of the myotome is associated with starvation, the use of myotome height / total length index has proved to be an effective measure of the condition of larvae (Koslow *et al.*, 1985); this index was used in the present study (§ 4.3.1.2).

Histological indices can also be used to survey larval condition. Starved fish larvae such as herring, plaice and turbot, were reported to have head shrinkage, reduction of the interorbital distance, a reduction of the liver or atrophy of the gut (in length and diameter) and desquamation of the intestinal mucosa (reviewed by Ferron & Leggett, 1994). This atrophy of the gut and liver is associated with a decrease of the height of the epithelial cells of the gut, liver and pancreatic cells. Severe starvation could cause the larvae to lose their digestive abilities and if the point-of-no-return is reached, larvae die even if food becomes available (Blaxter & Hempel 1963).

Behaviour

Behaviour may also be a useful tool in assessing the larval quality and is based on the fact that newly hatched larvae have specific behavioural patterns, which will be irregular or abnormal if larvae are weak or have a poor functionality. This was demonstrated on halibut and turbot larvae (Skiftesvik & Bergh, 1993); larvae in a 'bad' condition (e.g. bacteria-infected) were less active than those in better condition. Even though changes in behaviour are regarded as a sensitive measure of larval condition, there is no standardised method that actually correlates activity with a measure of viability. Furthermore, a link between larval growth and some behavioural variables was reported by Browman *et al.* (2002): cod larvae with higher growth rates moved shorter distances and had lower swimming speed, '*supporting the validity of applying these behavioural variables as proxies of larval quality-performance*'. The possibility of using phototactic response in quality assessment of larvae has also been recently suggested for halibut larvae (Karlsen & Mangor-Jensen, 2001).

Biochemical indicators

Buckley (1984) first used the RNA/DNA ratio as an index to measure the nutritional condition and growth of fish larvae. This ratio is based on the concept that the amount of DNA per cell is relatively constant, while the amount of RNA varies with physiological status, the requirement for protein synthesis, and growth. Rapid declines in RNA content and concentration and RNA/DNA ratio occur in starved larvae (Buckley *et al.*, 1999). Since then, it has been successfully used in a wide range of both marine and freshwater species (reviewed by Ferron & Leggett; 1994).

Work by Hakanson (1989) and Fraser (1988) indicated that lipid class composition was a reasonable measure of nutritional condition. The triacylglycerol (TAG; lipid reserves) content has been shown to be dependent on the nutritional state of the larvae while sterol (ST; structural lipids) content remains essentially unchanged during starvation; a nutritional index based on the TAG:ST ratio has been widely used since (e. g. Amara & Galois, 2004).

Chymotrypsin is a protease produced in the pancreas and secreted into the lumen of the intestine. It is responsible for the hydrolysis of peptide bonds in tyrosine, tryptophan, phenylalanine and methionine (Stryer, 1988) and its activity was recently characterised during the early ontogeny of red drum larvae (Applebaum *et al.*, 2001). The potential of this enzyme as an indicator of nutritional condition in marine larvae was tested by Applebaum & Holt (2003) after studying the response of chymotrypsin activity to food deprivations and reductions in nutrient intake. Thus, this enzyme activity was extremely low in larvae deprived of food and in larvae fed poor-quality live prey (starved rotifers).

In summary, as with egg quality, many parameters and methods have been proposed to determine larval quality. However none of them by itself appears to be enough to fully characterise it and hence, a combination of them gives more satisfactory results.

4.2 Aims

The aim of this part of the project was to assess the potential effects of nucleotide-enhanced broodstock diets on the larval quality of Atlantic halibut and haddock throughout 2001 and 2002-2003 spawning seasons respectively. Based on the proved efficacy of nucleotides when fed directly to larvae (§ 1.4.4.5) the effects of providing nucleotides through the broodstock diet upon larval morphometrics, growth, survival and first-feeding success were assessed.

4.3 Material and Methods

All the protocols followed to assess larval parameters are described in this section. Larval incubation protocols, morphometrics, performance and first feeding success of halibut and haddock larvae are included.

4.3.1 Halibut

Halibut larval experiments were carried out in 2001 with the aim of studying possible differences in the morphometrics and survival of larvae from eggs spawned by females fed the nucleotide-enriched and the control diets.

4.3.1.1 Larval incubation

Halibut eggs hatched at 75 – 80 ° days (degree days), when incubated at 6 °C approx., in the 70-L black incubators. Larvae have a big yolk-sac at hatch which is absorbed in less than 50 days post-hatching (Blaxter *et al.*, 1983; Pittman *et al.*, 1987). Since the number of yolk-sac tanks was limited when compared to the amount of egg batches collected and furthermore the yolk-sac stage is so lengthy, it was impossible to incubate all of them. It was therefore, necessary to make use of small-scale incubation systems. Lein & Holmefjord (1992) and Cutts (*pers. comm.*) succeeded using glass bowls for incubating halibut larvae throughout the yolk-sac stage. Therefore, due to their easy handling and replicability, 3-L glass bowls were used to follow the development of larvae until the yolk sac was fully absorbed. Immediately after eggs hatched, approximately 200 larvae from each incubated batch were transferred from the incubation tanks into a 3-L glass bowl filled with clean UV-sterilised, 5 μm filtered seawater at 5.5 – 6 °C and 35.5 ‰ salinity. Bowls containing larvae were then kept in a temperature-controlled room at 5.5 °C and complete darkness until yolk absorption was completed at 252 ° days. Half of the water volume in the bowl was carefully exchanged every 3 - 4 days. Half way through the yolk sac

stage (126 ° days), larvae were sampled for adenine nucleotide (AN) content and morphometric analyses.

4.3.1.2 Morphometric analysis

Larvae from each incubated batch were sampled for morphometric analysis at three different developmental stages:

- At hatch,
- Half-way through the yolk-sac stage (i.e. 126 ° days post-hatch), and
- At the end of yolk absorption (252 ° days post-hatch).

At least five images of individual larvae were captured with the same system previously used to measure egg size (§ 3.3.2.3).

The following parameters were considered:

a) **Total length** from mouth to the end of the primordial fin at the three developmental stages mentioned above.

b) **Myotome height** (larval depth, measured posterior to the anus). This was measured at 126 ° days and 252 ° days, but not at hatch due to the small size and poor image resolution.

c) **Yolk-sac volume**: Since the yolk-sac of the larvae has an ellipsoidal shape, the length and height of the sac were measured at hatch and 126 ° days, in order to calculate yolk-sac volume and subsequently yolk absorption rates. A set of x-y axes was overlain on the yolk-sac to accurately measure the length and maximum height of the yolk sac.

4.3.1.2.1 Morphometric calculations

Yolk-sac volume and subsequently yolk-sac absorption rates were then calculated by means of the following equations:

$$YSV = \frac{\pi}{6} L \times H^2, \qquad \textbf{[Eq.4. 1]} \text{ (Blaxter \& Hempel, 1963)}$$

where:

YSV is yolk-sac volume expressed in microlitres (µl)

L is yolk-sac length in millimetres.

H the height of the yolk-sac in millimetres.

$$YSAB = \frac{YSV_{hatch} - YSV_{126}}{t}, \quad \textbf{[Eq.4. 2]}$$

where:

YSAB is yolk-sac absorption rate expressed in microlitres per day (µl·day $^{-1}$).

YSV_{hatch} and YSV_{126} are the yolk sac volume at hatch and 126 ° days respectively.

t is time in days.

Length growth rate (***LGR***) of the larvae was calculated according to the following equation:

$$LGR = \frac{L_{252} - L_{hatch}}{t}, \quad \textbf{[Eq.4. 3]}$$

where:

L_{252} and L_{hatch} are larval length at 252 ° days and hatch respectively

t is time in days.

Condition of the larvae from each batch was assessed at 126 and 252 ° days using the following equation (Koslow *et al.*, 1985):

$$LK = \frac{MH}{L} \times 100, \quad \textbf{[Eq.4. 4]}$$

LK: larval condition.

MH: larval myotome height.

L: larval length.

4.3.1.2.2 Larval dry weight

Halibut larval samples were also taken for dry weight measurements at hatch and 252 ° days. Five larvae from each batch were placed inside an eppendorf tube, freeze vacuum dried and the dry weight measured on a scale. This operation was done in triplicate.

4.3.1.3 Larval performance assessment:

Dead halibut larvae were siphoned from each bowl with a 3-ml plastic pipette and under a very dim blue light. This operation was repeated every 3-4 days and mortalities recorded. At the end of the yolk-sac stage, live larvae were counted and therefore survival rate at the end of yolk-sac stage estimated for each batch. Larval samples were also taken for dry weight, morphometric and AN content analysis at 252 °days.

4.3.2 Haddock

Haddock larval experiments were carried out for two consecutive seasons in 2002 and 2003 in order to study possible differences in the morphometrics; survival of larvae from eggs from females fed the nucleotide-enriched and the control diets until the end of the yolk-sac stage in 2002, and until 10 days post hatch (10 DPH), when first feeding had already started, in 2003. Additionally, potential differences in gut development and first feeding success between control and nucleotide larvae where also studied during the second haddock spawning season.

4.3.2.1 Larval incubation

Haddock eggs hatched at circa 100 ° days (degree days), or 12.5 days at 8 °C approx., in 70-L incubators. Larvae hatch with yolk-sacs much smaller than those of halibut larvae so therefore the yolk is absorbed in 4-5 days post-hatching at 8 °C. Due to the limited incubation facilities

compared to the amount of egg batches collected -six per day (i.e. one per tank)-, it was also necessary to make use of small-scale incubation systems. Two different protocols were followed in 2002 and 2003: the glass bowl technique and a plastic vessel system.

4.3.2.1.1 Glass bowl technique

After the success of the glass bowl technique with halibut in the previous year, a similar approach was followed in 2002 with adjustments of the protocol and times to haddock biology. Eggs were left to hatch in the incubators tanks and approximately 200 larvae from each incubated batch were transferred into a 3-L glass bowl. Bowls were filled with clean UV-sterilised, 5 µm filtered seawater at 8 – 9 °C and 34-35 ‰ salinity. Bowls containing larvae were then kept in a temperature-controlled room at 8 °C and 100 lux. illumination.

4.3.2.1.1.1 Larval performance assessment:

The yolk-sac stage of haddock larvae is very short, so five days after hatching, when yolk was nearly absorbed, each bowl was checked, live larvae counted and survival rate for each batch estimated. Larval samples were also taken for dry weight, morphometric and adenine nucleotide (AN) content analyses at this stage.

4.3.2.1.2 Plastic vessels system

Some batches of larvae were incubated in 2002 using bowls with "*greenwater*" techniques and added rotifers until the beginning of the first feeding stage, but unsuccessfully since all the larvae died. Therefore, a different system was used to incubate the haddock larvae until 10 DPH in 2003, instead of glass bowls. Nine-litre plastic vessels (blue colour, polypropylene bucket shaped) were filled with 9 L of UV-sterilised, 5 µm filtered seawater at 8 – 9 °C and 34 - 35 ‰ salinity and 50 ml of *Nannochloris atomus* algae (to provide a vessel density of 0.5 million cells per ml). Aeration was placed at the centre of the bottom to provide sufficient water movement and prevent prey from settling out.

Haddock larvae were left to hatch in the egg incubation tanks and approx. 270 larvae were sampled using a small glass beaker and transferred into the 9-L plastic vessels. Stocking density was approximately 30 larvae per litre. Three vessels were set up for each batch and placed in a temperature-controlled room at 8 – 9 °C. Illumination was set up at 200 lux and increased to 500 lux on the fifth day (P. Smith, *pers. comm.*).

Brachionus plicatilis rotifers were used as live feed and were first introduced on the day after stocking at a density of five rotifers per ml (Downing & Litvak, 2001). Approximately 45,000 rotifers were added per vessel every day. Before adding new rotifers water subsamples were collected daily from each vessel and residual rotifer numbers assessed in order to check if rotifers numbers were decreasing. Water was exchanged every third day. Haddock larvae were incubated in this system until 10 DPH.

4.3.2.1.2.1 Larval performance and first feeding evaluation:

On the 10th day after hatching the experiment was terminated. Live and dead larvae from each vessel were counted in order to calculate survival rates. Feeding success was measured by checking the presence/absence of rotifers in the larval guts **(**Figure 4. 1**C)** under a binocular microscope.

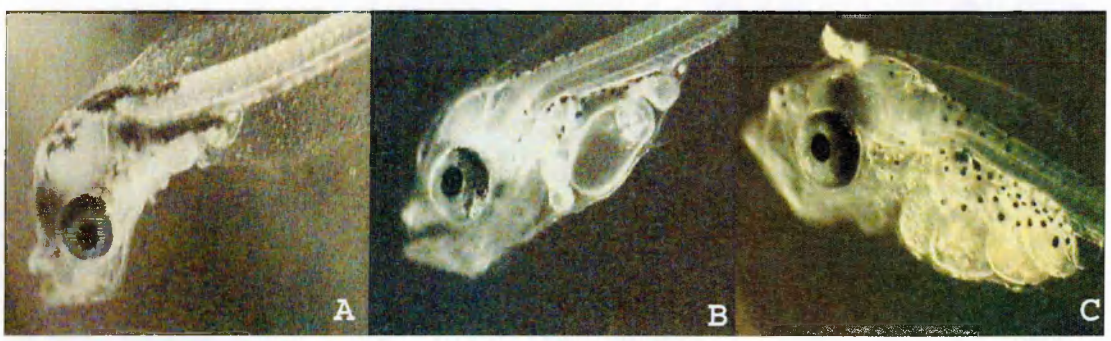


Figure 4. 1 Gut development in haddock larvae at 10 days post hatching (DPH); A) Non-, B) Half-, and C) Advanced developed.

The degree of gut development at 10 DPH was assessed from the images taken for morphometric analysis (§ 4.3.2.2). Based on the description of haddock gut development

(Hamlin *et al.* 2000), larval images were classified according to their gut developmental stage, into one of the following categories: non, medium and advanced developed:

- **Non-developed**: intestine is not developed, no convolutions observed (Figure 4. 1A).
- **Medium-developed**: intestine can be observed and a constriction separates the mid-intestine from the hindgut (Figure 4. 1B).
- **Advanced-developed**: intestine became much wider and with multiple convolutions (Figure 4. 1C).

4.3.2.2 Morphometric analysis

Haddock larvae from each incubated batch were sampled for morphometric analysis at three different developmental stages:

- At hatch in 2002 and 2003.
- At the end of yolk absorption (5 DPH) in 2002.
- At 10 DPH in 2003.

At least five larval images per batch were captured using the same system previously used to measure egg size (§ 3.3.2.3) and halibut larvae (§ 4.3.1.2).

The parameters measured were the same previously used and defined in section 4.3.1.2 for halibut larvae:

d) **Total length** at the three developmental stages mentioned above

e) **Myotome height** measured at the three stages.

f) **Yolk-sac volume** at hatch.

4.3.2.2.1 Morphometric calculations

Yolk-sac volume was calculated according to equation 4.1 (Blaxter & Hempel, 1963). Yolk sac absorption rate was estimated as follows:

$$YSAB = \frac{YSV_{hatch}}{t}, \qquad \textbf{[Eq.4. 5]}$$

where:

YSAB is yolk-sac absorption rate expressed in microlitres per day ($\mu l \cdot day^{-1}$).

YSV_{hatch} is the yolk sac volume at hatch, ***t*** is time expressed as the number of days until yolk is absorbed.

Length growth rate (***LGR***) was calculated according to equation 4.3 but using larval length at 5 (L_5) and 10 DPH (L_{10}) in 2002 and 2003 respectively instead of L_{252}. Condition of the larvae from each batch was assessed at hatch, 5 and 10 DPH according to equation 4.4.

4.3.2.2.2 Larval dry weight

Haddock larval samples were also taken for dry weight at hatch (in 2002 and 2003), 5 DPH (only 2002) and 10 DPH (only 2003). Five larvae from each batch were placed inside an eppendorf tube, freeze vacuum dried and dry weight measured on a scale. This operation was done in triplicate.

4.3.3 Data analysis

Data analysis was carried out following the procedures explained in section 2.3.6. Since the egg batches were collected on different dates along the spawning season and egg size decreases over the season, larval length and myotome height of the two dietary groups were divided by egg diameter and yolk-sac volume by egg volume to control for differences in egg size. The resulting parameters were then analysed as explained above (§ 2.3.6). The effects of spawning biomass, batch, egg and larval characteristics together with diet on the survival and first feeding success were assessed using general linear models with diet as a class variable using the SAS procedure GLM (SAS Institute, Inc. 1988). Batch characteristics included mass, number of eggs, proportion of eggs that were floating, density, fertilisation rate (FR) and hatching rate (HR). Egg characteristics were size, wet and dry weight and specific gravity. Larval characteristics included length (L), myotome height (MH), yolk-sac volume and condition at hatch, 5 DPH and 10 DPH, and percentages of non- and developed-guts. Scores for gut development were analysed statistically using a Chi-square (χ^2) test contingency table.

4.4 Results

4.4.1 Halibut Trial

4.4.1.1 Larval morphometrics

At the end of the halibut spawning season 25 egg batches were incubated until the end of the yolk-sac stage (11 were control and 14 nucleotide). Halibut larvae were incubated until the end of the yolk-sac stage (252 ° days) and the morphometric measures were taken at hatch, half-way (126 ° days) and end of the yolk-sac stage.

At hatch:

Larvae from broodstock fed the control diet were larger than those from broodstock fed the nucleotide-enhanced diet (from now onwards referred to as control larvae and nucleotide larvae respectively) in terms of length (C: 5.9440 ± 0.0519 mm, NT: 5.7683 ± 0.0393; p = 0.007) and dry weight (1.2441 ± 0.0231 mg vs. 1.1667 ± 0.0213 mg; p = 0.017), although yolk sacs at hatch were of similar size (C: 15.783 ± 0.557 µl vs. NT: 14.945 ± 0.517 µl; p = 0.2568) (Table 4.1). Unfortunately, myotome height at hatch could not be measured accurately enough as a result of the poor image resolution; therefore, condition at hatch was calculated based on the larval dry weight and length (dry weight / length ^{3}). Nevertheless, the condition of the nucleotide larvae was not compromised (C: 0.0061 ± 0.0003; NT: 0.0062 ± 0.0004; p = 0.813) by their smaller size (Table 4. 1). Furthermore, when larval lengths at hatch were corrected for egg size, the difference was not significant (F = 1.59; p = 0.220), indicating that nucleotide larvae were significantly smaller than controls only as a result of nucleotide eggs being significantly smaller (chapter 3). The same occurred when larval dry weight at hatch was corrected by egg dry weight, with no significant difference between both dietary treatments (F = 1.38; p = 0.253).

Table 4. 1 Halibut larval morphometrics of control and nucleotide diets from 2001 spawning season at hatch, 126 °days and 252 °days. Significant differences between diets are denoted by *. (Mean ± SEM).

Parameters	Control	Nucleotide
At hatch		
Length (mm)	5.9440 ± 0.0519 *	5.7683 ± 0.0393
Myotome height (mm)	N/A	N/A
Yolk-sac volume (µl)	15.783 ± 0.557	14.945 ± 0.517
Dry weight [0] (mg)	1.2441 ± 0.0231 *	1.1667 ± 0.0213
K - condition [1]	0.0061 ± 0.0003	0.0062 ± 0.0004
At 126 degree days		
Length (mm)	10.463 ± 0.100	10.545 ± 0.108
Myotome height (mm)	0.6459 ± 0.0086	0.6424 ± 0.0111
Yolk-sac volume (µl)	3.372 ± 0.153 *	2.649 ± 0.104
K - condition [2]	0.0622 ± 0.0007	0.0603 ± 0.0001
At 252 degree days		
Length (mm)	12.624 ± 0.094	12.402 ± 0.105
Myotome height (mm)	0.7912 ± 0.0075	0.7694 ± 0.0073
Dry weight (mg)	1.2088 ± 0.0395	1.1508 ± 0.0440
K - condition [2]	0.0624 ± 0.0014	0.0622 ± 0.0010
Length growth rate [3]	0.1596 ± 0.0046	0.1601 ± 0.0048

[0]: including yolk sac;
[1]: dry weight / length ^{3} ;
[2]: myotome height / length;
[3]: mm / day

The variation of larval size at hatch was studied throughout the season but no significant correlation appeared with any of the seasonal variables tested (date, broodstock tank temperature or batch number). However, the volume of the yolk sacs at hatch became smaller with the season and larvae that hatched earlier in the season had bigger yolk sacs (Figure 4. 2); although the diet effect was not significant ($F = 0.03$; $p = 0.855$) after an ANCOVA test was carried out (Table 4. 2).

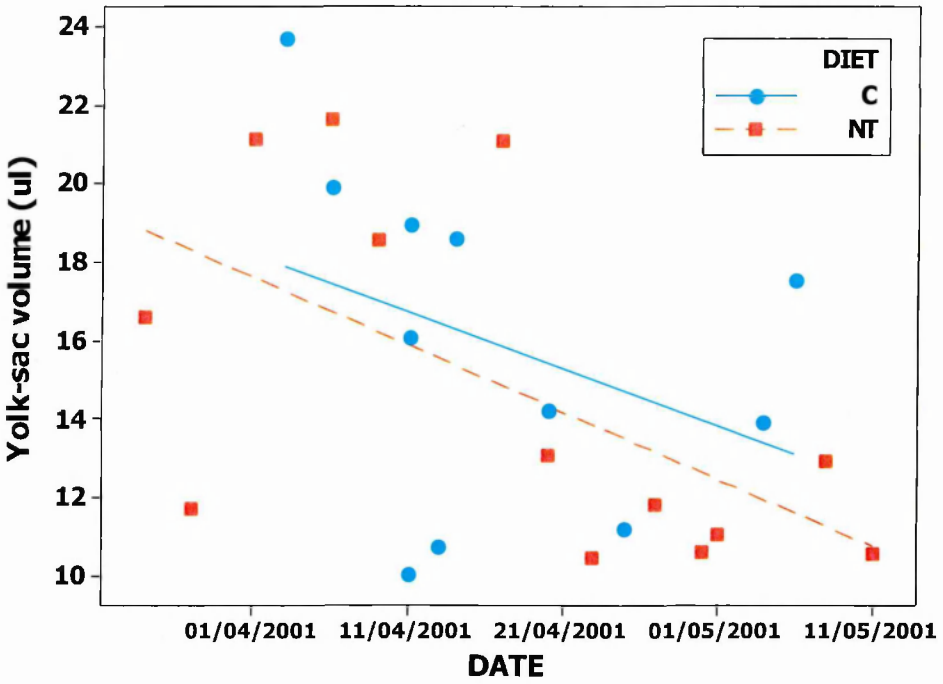


Figure 4. 2 Scatterplot of the yolk-sac volume of Atlantic halibut larvae at hatch throughout the spawning season in 2001. Control (C: blue circles) and Nucleotide (NT: red squares).

Larval length and yolk-sac volume were not correlated to egg size ($p = 0.849$ and $p = 0.937$ respectively). Yolk-sac volume was positively correlated with blastomere morphology (BM) ($r = 0.4696$, $p = 0.0206$); the higher the BM, the larger the yolk sac at hatch. However, BM was not correlated with egg size ($r = -0.121$, $p = 0.407$). No significant correlation was observed between dry weights of eggs and larvae at hatch ($r = 0.273$, $p = 0.208$). However broodfish in a better condition produced heavier larvae (dry weight) ($r = 0.617$, $p = 0.002$). The importance of broodstock condition on drop-out rates and egg quality was reported in chapter 2 and 3 respectively.

Table 4. 2 ANCOVA analysis of yolk-sac volume of halibut larvae at hatch from 2001 spawning season. ** Denotes significant effect. 'Diet*date' means interaction between diet and date. R^2-adj = 16.52 %

	DF	Sums of Squares	Mean Square	F Value	P Value
Diet	1	0.54	0.54	0.03	0.855
Date	1	84.24	84.24	5.35	0.031**
Diet*Date	1	0.54	0.54	0.03	0.855
Error	20	314.78	15.74		

At 126 degree-days:

Larval length and myotome height were similar between the control and the nucleotide larvae at 126 ° days (p = 0.583 and p = 0.799 respectively; Table 4. 1). However, larval length and myotome height at this stage increased over the spawning season, meaning that larvae from later batches were larger than those from earlier batches at 126 °days (Figure 4. 3).

Yolk sac volume was considerably reduced by this stage, when compared to their initial size at hatch and remained significantly larger for the control larvae throughout the season (F = 16.05, p = 0.000; Figure 4. 3). Myotome height was positively correlated to larval length (r = 0.5581, p = 0.0069) and yolk-sac volume at this stage (r = 0.5004, p = 0.0177), although length was not correlated with yolk-sac volume.

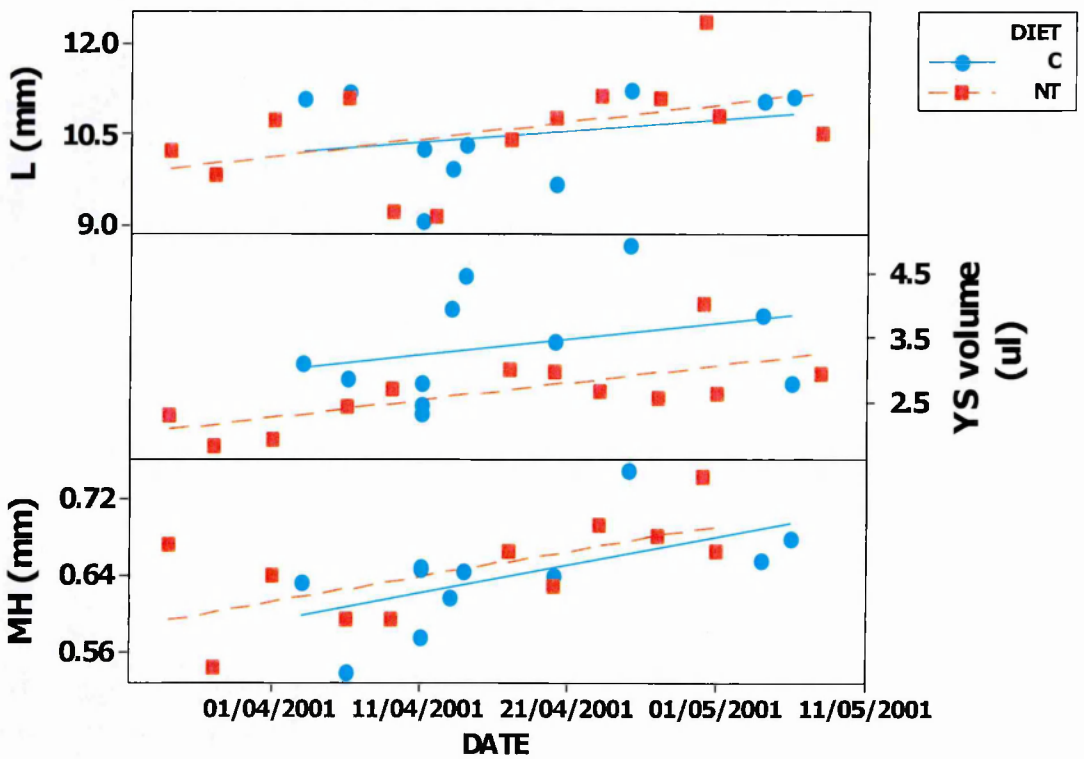


Figure 4. 3 Scatterplots of the length (L), yolk-sac (YS) volume and myotome height (MH) of halibut larvae at 126 °days from 2001 spawning season. Control (C: blue circles); nucleotide (NT: red squares).

Since egg size was significantly different between both diets (§ 3.4.1), lengths and myotome height were divided by egg diameter, and yolk-sac volume by egg volume. Nucleotide larvae

were then significantly larger than those from the control group when length was corrected for the egg size (F = 4.82, p = 0.039; Figure 4. 5). Corrected myotome height was not significantly different between diets (F = 2.31, p = 0.122) although expected, nor yolk-sac volume (F = 0.03, p = 0.872). Condition of the control and nucleotide larvae was also similar (Table 4. 1).

Table 4. 3 ANCOVA analysis of yolk-sac volume of halibut larvae at 126 °days from 2001 spawning season. * Denotes significant effect. The interaction diet and date was removed since it was not significant. R^2-adj = 38.33 %

	DF	Sums of Squares	Mean Square	F Value	P Value
Diet	1	0.0021	0.0021	7.22	0.014*
Date	1	0.0023	0.0023	8.04	0.010*
Error	20	0.0057	0.00028		

At 252 degree-days:

At the end of the yolk-sac stage there were no significant differences between control and nucleotide larvae in any of the parameters tested (i.e. length, myotome height, dry weight and condition Table 4. 1). Typical halibut larvae at this stage were 12.5 mm long, with a myotome height of around 0.8 mm and weighed 1.2 mg (dry weight).

Separate GLMs were used to test for diet, tank, female or date effects on length, myotome height and dry weight but none of them were significant. Instead, both larval length and myotome height at the end of the yolk-sac stage were correlated to the condition of the female (r = 0.4658, p = 0.016; r = 0.6525, p = 0.000 respectively), although egg size was not (p = 0.924). Larval myotome height at this stage increased with length at hatch (r = 0.5143; p = 0.010) and at 252 ° days (r = 0.3960; p = 0.05).

Dry weight of the larvae did not differ significantly between dietary treatments at this stage (F = 0.94; p = 0.337; Table 4. 1). This was confirmed by carrying out a GLM that revealed that diet, tank, female or date effects were not significant (F = 0.00, p = 0.968; F = 0.48, p = 0.751; F =

1.42, p = 0.298; and F = 0.01, p = 0.934 respectively; Table 4. 4). Data from both dietary treatments were then pooled and a quadratic relationship with date was found (F = 4.74; p = 0.041; Figure 4. 4).

Table 4. 4 General linear model (GLM) of larval dry weight at 252 °days of Atlantic halibut from the 2001 spawning season. None of the terms of the model were significant.

	DF	Sums of Squares	Mean Square	F Value	P Value
Diet	1	0.00005	0.00005	0.00	0.968
Tank (Diet)	4	0.05621	0.01405	0.48	0.751
Female (Tank Diet)	7	0.29056	0.04151	1.42	0.298
Date	1	0.00021	0.00021	0.01	0.934
Error	10	0.29322	0.02932		

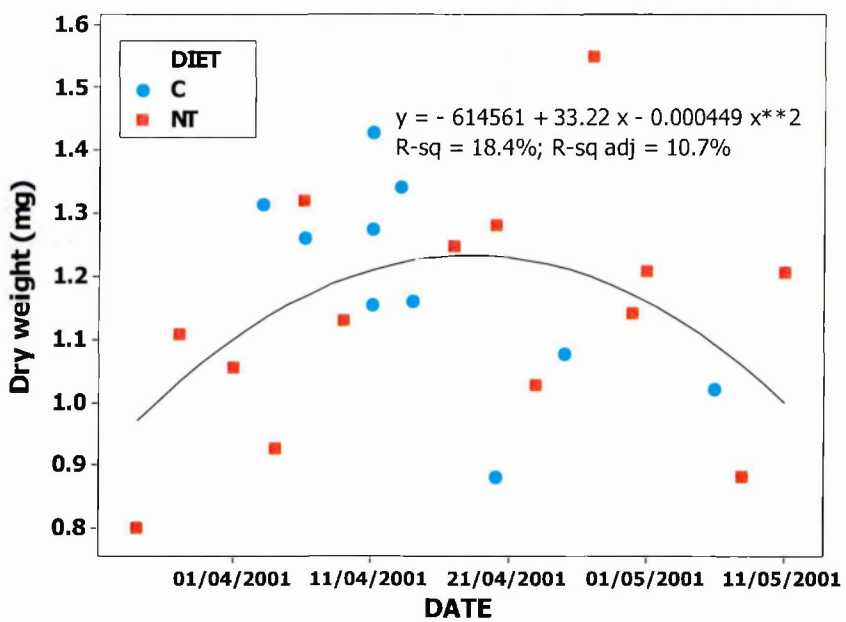


Figure 4. 4 Scatterplot of the dry weight of Atlantic halibut larvae at 252 °days throughout the spawning season in 2001. Control (C: blue circles) and Nucleotide (NT: red squares).

Larval dry weight at the end of the yolk-sac stage was also positively correlated with broodfish condition (r = 0.605; p = 0.002), blastomere morphology (r = 0.451; p = 0.027), and length at 252 °days (r =0.579; p = 0.003), and negatively correlated with total drop-out (r = - 0.443; p = 0.034). Correlations with fertilisation and hatching rates were almost significant (r = 0.365, p = 0.079; r = 0.403, p = 0.051 respectively).

The absorption rate of the yolk sac was measured after 126 °days from hatching. However similar values were reported between control and nucleotide larvae (C: 0.5973 ± 0.0674 µl / day, NT: 0.5896 ± 0.0643 µl / day; F = 0.01; p = 0.934).

Overall, growth rates were similar between both dietary treatments (F = 0.00, p = 0.945; Table 4. 1). However, when egg size differences were taken into account, nucleotide larvae grew significantly faster than control larvae over the yolk-sac period (Figure 4. 5). In other words, when control and nucleotide larvae of the same size were followed until the end of the yolk-sac stage, those from the nucleotide group grew faster than control larvae.

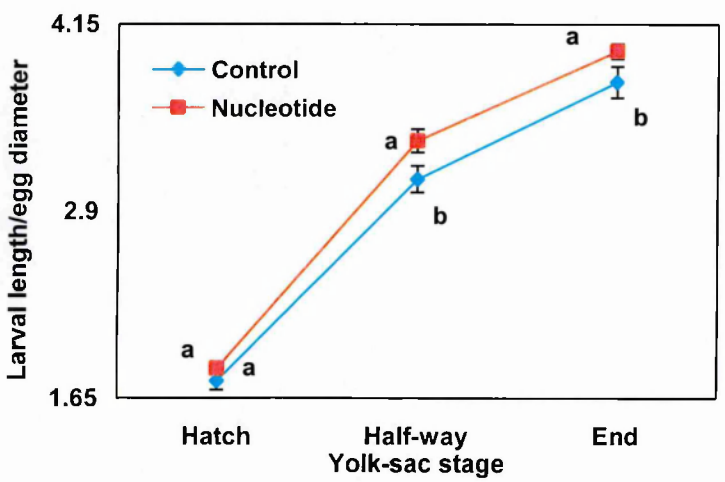


Figure 4. 5 Halibut larval growth during the yolk-sac stage for control and nucleotide diets in 2001 spawning season. Significant differences between diets are denoted with different letters. (Mean ± SEM).

Linear regression analysis showed that larval growth rate at 252 °days was negatively correlated to the length of larvae at hatch (Figure 4. 6), indicating that smaller larvae grew faster than bigger larvae (F = 5.65; p = 0.026).

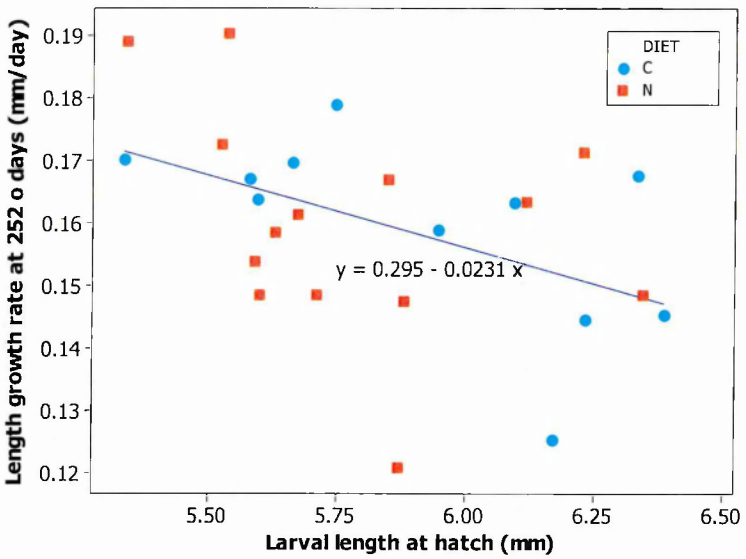


Figure 4. 6 Relationship between larval length at hatch and growth rate at 252 °days of halibut larvae from 2001 spawning season. Control (C: blue circles); Nucleotide (NT: red squares). R^2 = 19.7 %.

4.4.1.2 Larval performance

Survival

At the end of the yolk-sac stage (252 ° days), 35.08 ± 5.82 % of the stocked larvae from fish fed the control diet survived, compared with the 47.6 ± 8.20 % for the larvae from the nucleotide treatment. This difference was significant and in favour of the nucleotide diet (F = 4.64, p = 0.0459; Figure 4. 7). Survival rates were positively correlated with egg density (r = 0.4205, p = 0.0457) and length of the larvae at 252 °days (r = 0.4687, p = 0.0241), indicating that those batches in which larvae grew faster also presented a higher survivorship.

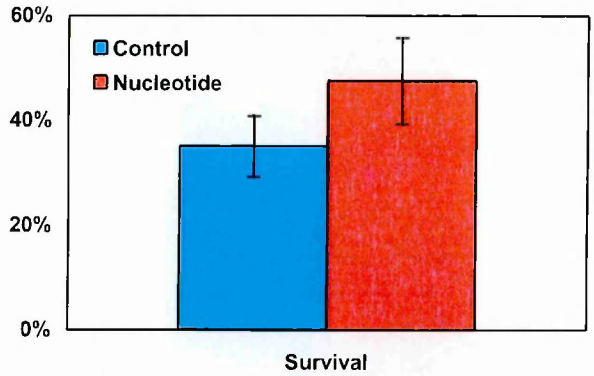


Figure 4. 7 Halibut larval survival at the end of the yolk-sac stage with both dietary treatments diets in 2001 spawning season. Difference between diets was significant (see text). (Mean ± SEM).

4.4.2 Haddock Trial

4.4.2.1 Larval morphometrics

As explained in section 4.3.2.1, haddock larvae were incubated after hatch using different systems in 2002 and 2003 depending on the time-scale of the trial. In the first year, the purpose of the trial was to study the survival and the morphometrics of the larvae until the end of yolk absorption (5 DPH) so larval samples were taken at hatch and 5 DPH. In 2003, survival and morphometrics were assessed at 10 DPH, once first feeding had started; larval samples were then taken at hatch and 10 DPH only.

First spawning season (2002)

Due to the large amount of egg batches collected, six per day (one per tank), and limited incubator availability, a total number of 30 batches were incubated in 2002, from which 15 were spawned by the control fish and the remaining were from the nucleotide tanks. Control and nucleotide larvae were not significantly different for any of the parameters studied at hatch in 2002 (Table 4. 5). Length (C: 4.2811 ± 0.0289 mm vs. NT: 4.2633 ± 0.0272 mm), myotome height (C: 0.2687 ± 0.0029 mm vs. NT: 0.2678 ± 0.0034 mm), yolk-sac volume (C: 0.4588 ± 0.0412 µl vs NT: 0.4923 ± 0.0645 µl), dry weight (C: 0.1905 ± 0.0193 mg vs. NT: 0.1929 ± 0.0125 mg) and larval condition (C: 6.2793 ± 0.0554 vs. NT: 6.2833 ± 0.0691) were very

similar at hatch between both diets. Size of the larvae at hatch (by length) increased with egg dry weight ($r = 0.6943$, $p = 0.0002$) and with egg size ($r = 0.6541$, $p = 0.0002$; Figure 4. 8) and therefore decreased throughout the season (§ 3.4.2.2, $r = - 0.5260$, $p = 0.004$). Myotome height (MH) at hatch was almost significantly negatively correlated with batch number ($r = - 0.3390$; $p = 0.0776$) indicating a possible decreasing trend throughout the season. This was confirmed since MH increased with egg size ($r = 0.4325$, $p = 0.0215$) and was highly correlated with larval size ($r = 0.8376$, $p < 0.0001$; Figure 4. 9). Similarly, yolk sac size was almost negatively correlated with date ($r = - 0.3403$, $p = 0.076$), and this trend was supported by the fact that yolk-sac volumes increased with egg wet and dry weight as well as with egg diameter ($r = 0.4802$, $p = 0.0097$; $r = 0.4633$, $p = 0.026$ and $r = 0.4053$, $p = 0.0324$ respectively). The size of the yolk sac did not appear to be correlated either to larval length nor to myotome height of the larvae at hatch. Larval dry weight at hatch increased with egg size ($r = 0.4434$, $p = 0.0181$) and decreased along the spawning season ($r = - 0.4257$, $p = 0.0213$).

Table 4. 5 Haddock larval morphometrics of control and nucleotide diets in 2002 and 2003 spawning seasons. Significant differences between diets are denoted by *. (Mean ± SEM).

	2002		2003	
Parameters	Control	Nucleotide	Control	Nucleotide
At hatch				
Length (mm)	4.2811 ± 0.0289	4.2633 ± 0.0272	4.1275 ± 0.0708	4.1740 ± 0.0269
Myotome height (mm)	0.2687 ± 0.0029	0.2678 ± 0.0034	0.2600 ± 0.0037	0.2633 ± 0.0073
Yolk-sac volume (µl)	0.4588 ± 0.0412	0.4923 ± 0.0645	0.2311 ± 0.0238	0.4976 ± 0.0405 *
Dry weight (mg)	0.1905 ± 0.0193	0.1929 ± 0.0125	0.2857 ± 0.0253	0.3200 ± 0.0413
K - condition [1]	6.2793 ± 0.0554	6.2833 ± 0.0691	6.3085 ± 0.0762	6.3080 ± 0.1670
At 5 DPH (2002) & 10 DPH (2003)				
Length (mm)	4.6928 ± 0.0252	4.7363 ± 0.0228	4.3913 ± 0.0429	5.1074 ± 0.0533 *
Myotome height (mm)	0.2945 ± 0.0023	0.2981 ± 0.0026	0.2500 ± 0.0033	0.2945 ± 0.0059 *
Dry weight (mg)	0.2041 ± 0.0166	0.2440 ± 0.016 *	0.3421 ± 0.0277	0.3611 ± 0.0205
K - condition [1]	6.2789 ± 0.0448	6.2996 ± 0.0568	5.703 ± 0.0600	5.7710 ± 0.1000
Length Growth rate (mm/day)	0.1403 ± 0.0174	0.1489 ± 0.0141	0.0422 ± 0.0033	0.0910 ± 0.0096 *

[1]: myotome height / length

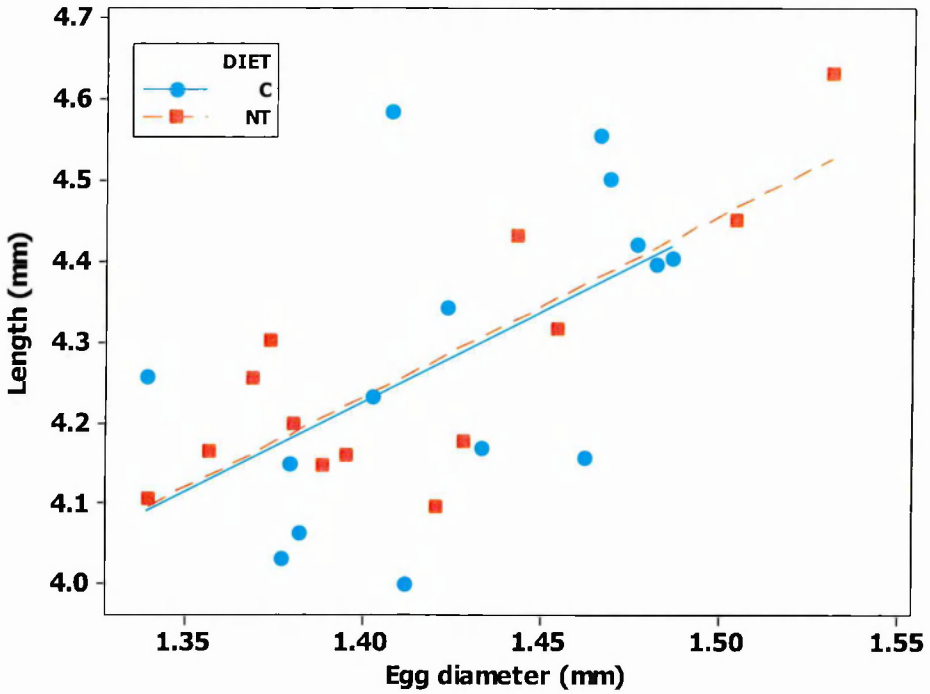


Figure 4. 8 Scatterplot of larval length at hatch versus diameter of haddock eggs from the 2002 spawning season. Control (C: blue circles); Nucleotide (NT: red squares).

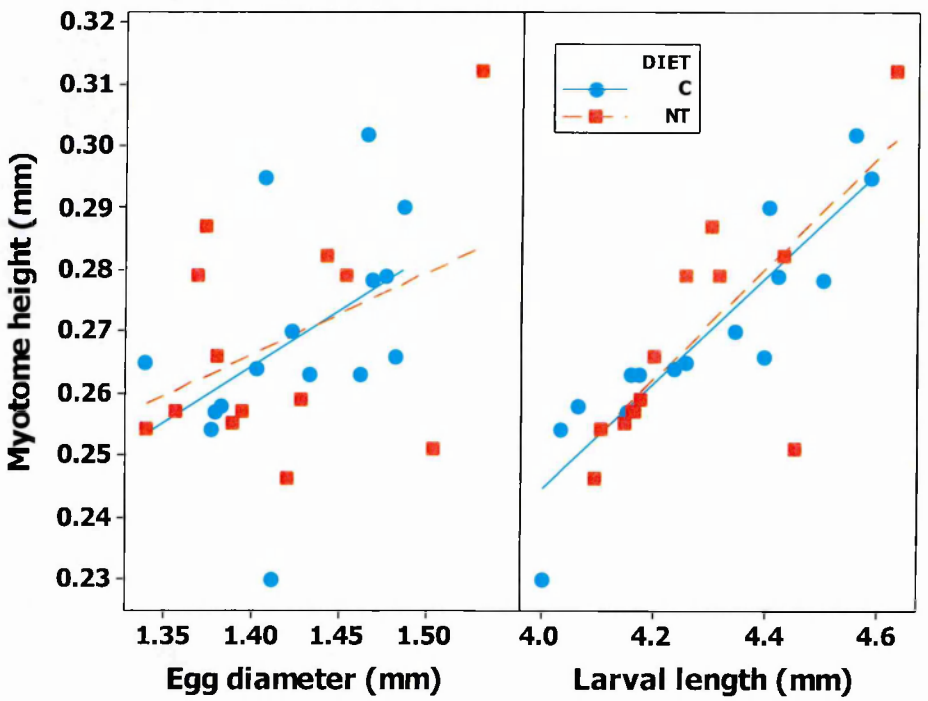


Figure 4. 9 Scatterplot of myotome height at hatch versus egg diameter and length at hatch of haddock larvae from the 2002 spawning season. Control (C: blue circles); Nucleotide (NT: red squares).

Five days later (during the 2002 season), most of the morphometric parameters remained similar for both diets. Control larvae were 4.6928 ± 0.0252 mm long, with a MH of 0.2945 ± 0.0023 mm whereas larvae from the 'nucleotide-fed' females were 4.7363 ± 0.0228 mm long, and with a MH of 0.2981 ± 0.0026 mm. However, the nucleotide larvae were significantly heavier in terms of dry weight (0.2440 ± 0.016 mg vs. 0.1905 ± 0.0193 mg) when compared to the control group at 5 DPH ($F = 10.04$, $p = 0.0048$; Table 4. 5). Larval length and MH at 5 DPH increased with egg wet weight and diameter and larval length at hatch. Myotome height at hatch and at 5 DPH were also correlated to length at 5 DPH, while MH at 5 DPH was increased with larger yolk-sac volume and with length at 5 DPH (Table 4. 6). No seasonal pattern of variation was found for length although MH followed an U-shape distribution with higher values during the first and last batches in the season ($F = 4.76$; $p = 0.019$; Figure 4. 10). The condition of the larvae at 5 DPH, which did not differ between diets (C: 6.2789 ± 0.0448, NT: 6.2996 ± 0.0568; $F = 0.08$, $p = 0.774$), increased with egg dry weight and the volume of the yolk sac at hatch (Table 4. 6; Figure 4. 11).

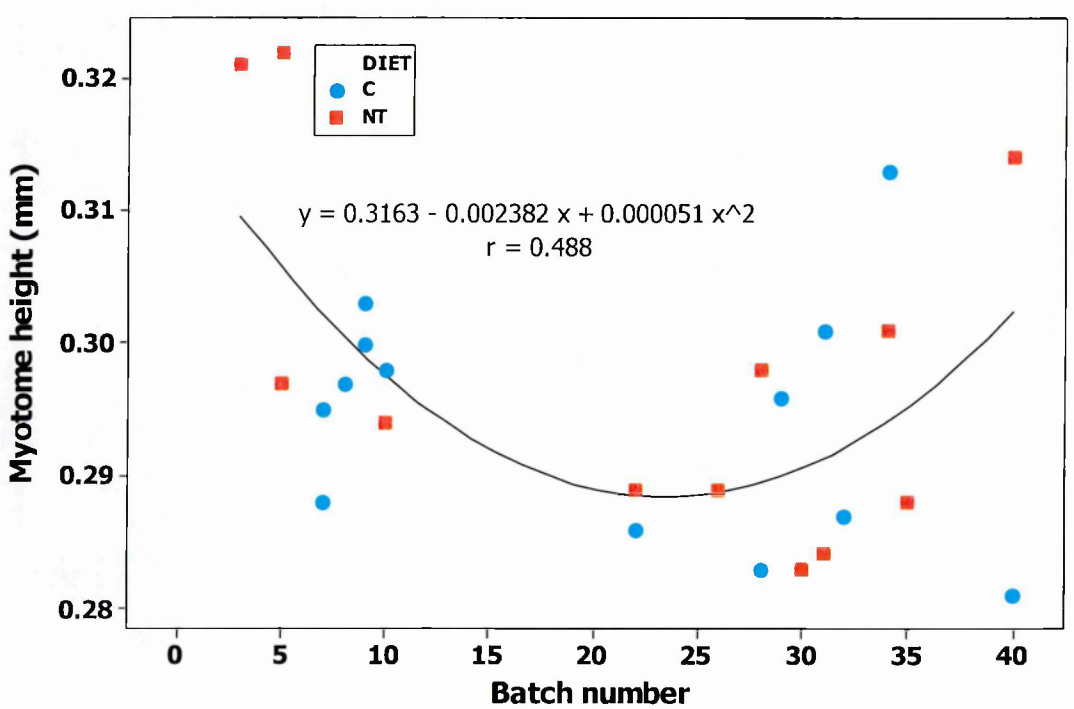


Figure 4. 10 Variation of the myotome height of haddock larvae at 5 days post hatching (DPH) during the 2002 spawning season. Control (C: blue circles); Nucleotide (NT: red squares).

Table 4. 6 Correlations between egg and larval parameters at hatch and 5 days post hatching (DPH) of haddock broodstock from the 2002 spawning season. Significant correlations are in bold format.

Cell contents:	r - value / p - value	Egg			Larvae at hatch					Larvae at 5 DPH		
		Dry weight	Wet weight	Diameter	Dry weight	Length	MH	YS Volume	Condition	Dry weight	Length	MH
Egg	Wet weight	**0.390** **0.000**										
	Diameter	**0.282** **0.005**	**0.473** **0.000**									
Larvae at hatch	Dry weight	0.363 0.075	0.262 0.170	**0.443** **0.018**								
	Length	**0.694** **0.000**	**0.526** **0.004**	**0.654** **0.000**	0.093 0.650							
	MH	0.317 0.141	0.296 0.126	**0.432** **0.022**	- 0.046 0.823	**0.837** **0.000**						
	YS Volume	**0.463** **0.026**	**0.480** **0.010**	**0.405** **0.032**	0.349 0.081	0.237 0.224	0.083 0.674					
	Condition	- 0.168 0.444	- 0.050 0.800	0.048 0.808	- 0.168 0.411	**0.389** **0.041**	**0.828** **0.000**	- 0.101 0.610				
Larvae at 5 DPH	Dry weight	0.162 0.451	0.273 0.160	0.121 0.539	0.130 0.508	- 0.135 0.511	- 0.193 0.346	- 0.014 0.947	- 0.191 0.351			
	Length	0.364 0.105	**0.495** **0.012**	**0.521** **0.009**	0.249 0.240	**0.418** **0.047**	**0.441** **0.035**	-0.032 0.883	0.300 0.164	0.049 0.824		
	MH	**0.705** **0.000**	**0.625** **0.001**	**0.604** **0.002**	0.354 0.090	**0.444** **0.034**	0.299 0.166	**0.433** **0.039**	0.049 0.826	- 0.008 0.972	**0.522** **0.007**	
	Condition	**0.554** **0.009**	0.377 0.063	0.328 0.118	0.228 0.284	0.222 0.308	0.037 0.868	**0.527** **0.010**	- 0.154 0.482	- 0.042 0.850	- 0.122 0.562	**0.782** **0.000**

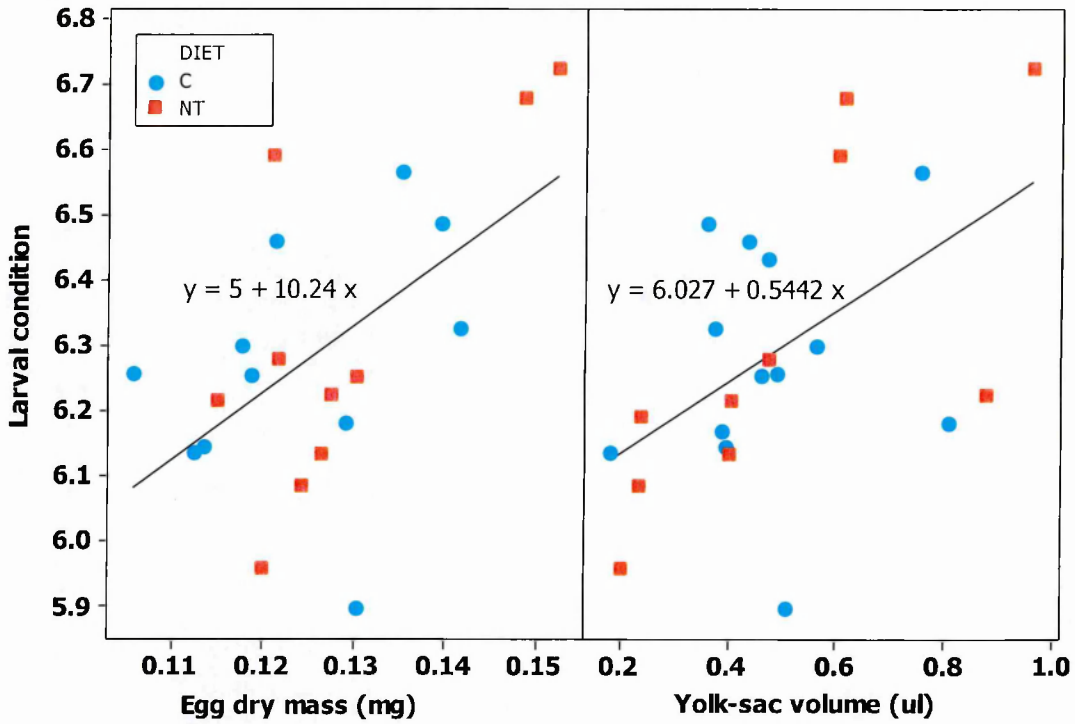


Figure 4. 11 Scatterplot of larval condition at 5 days post hatching (DPH) against the egg dry weight and the yolk-sac volume of haddock larvae from both dietary treatments in the 2002 spawning season. Control (C: blue circles); Nucleotide (NT: red squares).

At this stage (5DPH) and after a one-way ANOVA analysis the nucleotide larvae were not significantly heavier (0.2440 ± 0.016 mg vs. 0.2041 ± 0.0166 mg; $F = 2.99$, $p = 0.096$) than control larvae (Figure 4. 12). However, a GLM analysis showed a dome-shape distribution, with nucleotide larvae from the beginning and middle of the spawning season being significantly heavier than those from the control group ($F = 10.04$, $p = 0.0048$, Table 4. 7,

Figure 4. 13). However, larval dry weight at 5 DPH was not correlated to egg diameter ($p = 0.5393$) despite the latter decreasing along the season (Chapter 3). Length growth rates during the period of yolk sac absorption were similar between diets (Table 4. 5) however the weight gain was significantly higher in the nucleotide larvae (Figure 4. 12).

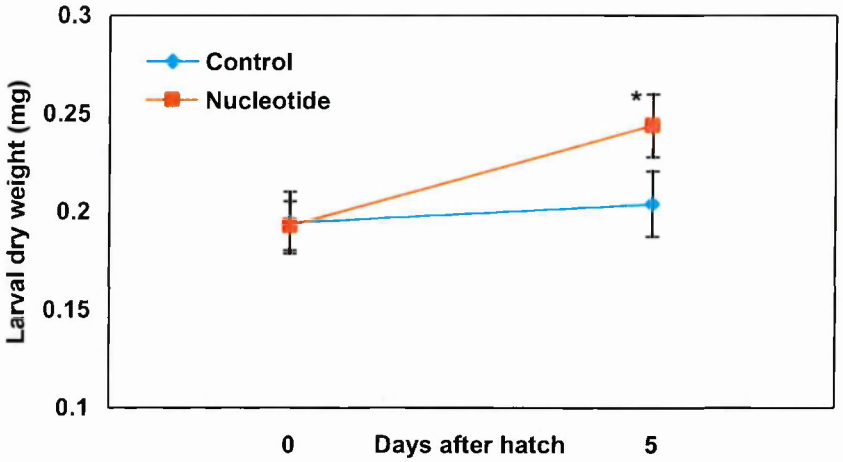


Figure 4. 12 Haddock larval dry weight during the yolk-sac stage for control and nucleotide diets in 2002 spawning season. Significant differences between diets are denoted with *. (Mean ± SEM).

Table 4. 7 GLM analysis on haddock larval dry weight at 5 days post hatch (DPH) from broodstock fed two different diets (control and nucleotide) in 2002. * denotes significant differences.

Variation Source	DF	Sums of Squares	Mean Squares	F Value	P Value	R^2	R^2 –adj
Total	7	0.06485	0.00926	4.33	0.0046*	60.25	46.34
Diet	1	0.02149	0.02149	10.04	0.0048*		
Tank (diet)	4	0.01988	0.00497	2.32	0.0919		
Batch Number	1	0.01927	0.01927	9.01	0.0071*		
Batch Number^2	1	0.02995	0.02995	14.00	0.0013*		
Error	20	0.04279	0.00214				

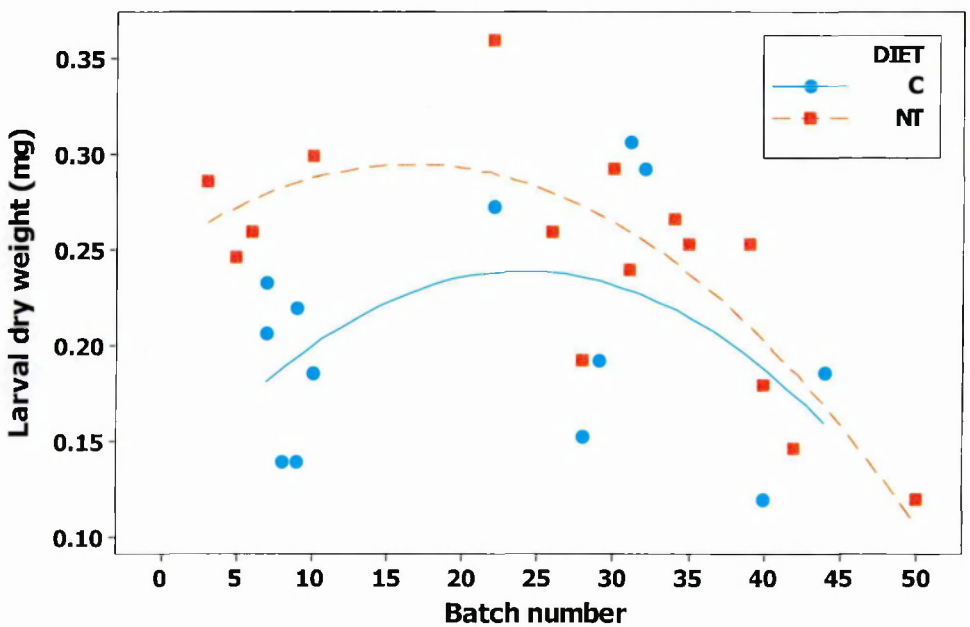


Figure 4. 13 Variation of the dry weight of haddock larvae at 5 days post hatch (DPH) along the spawning season in 2002. Control (C: blue circles); Nucleotide (NT: red squares).

Second spawning season (2003)

In the second spawning season (2003), three egg batches from each dietary treatment were incubated and the larval morphometric parameters were measured at hatch and 10 DPH, when the first feeding had already started. No differences were found in length, myotome height, condition and dry weight between control and nucleotide larvae at hatch (Table 4. 5). However, nucleotide larvae had a significantly larger yolk-sac volume at this stage as a result of the NT group producing significantly bigger eggs (0.4976 ± 0.0405 µl vs. 0.2311 ± 0.0238 µl; $F = 36.96$, $p = 0.000$; Table 4. 5). Larval length at hatch was not correlated with seasonal variables such as date, broodstock water temperature or batch number. However, when comparing length with the mean egg diameter for each batch it was positively correlated with egg size ($R^2 = 99.40$ %, $p = 0.002$; Figure 4. 14). Bigger larvae, in terms of length, exhibited larger myotome heights ($r = 0.521$, $p = 0.011$), and were therefore also in better condition but there was no relation with yolk-sac volume ($p = 0.683$).

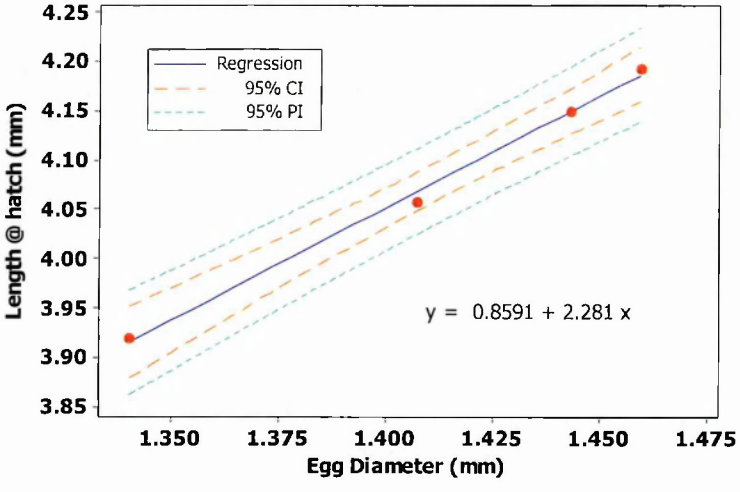


Figure 4. 14 Relationship between larval length at hatch and egg diameter of haddock eggs in the second spawning season (2003).

Yolk-sac volume was negatively correlated with date ($r = - 0.661$, $p = 0.001$) and batch number (BN) ($r = - 0.827$, $p = 0.000$), but was positively correlated with egg size ($r = 0.854$, $p = 0.000$). Yolk-sac volume therefore, varied with egg size which in turn decreased along the season (Chapter 3). When yolk-sac volume was divided by egg volume and compared between both dietary groups, significant differences were found ($F = 18.12$, $p = 0.001$), hence the yolk sacs of the nucleotide larvae were larger than expected.

At 10 DPH, nucleotide larvae were significantly bigger in terms of length (5.1074 ± 0.0533 mm vs. 4.3913 ± 0.0429 mm; F = 110.46, p = 0.000) and myotome height (0.2945 ± 0.0059 mm vs. 0.2500 ± 0.0033 mm; T = - 6.59, p = 0.000) when compared with control larvae. Bigger eggs, however, also produced larger larvae in terms of length (r = 0.9293, p = 0.0223) and myotome height (r = 0.9161, p = 0.0288). Since egg size differences were also considered the larval length and myotome height at 10 DPH were divided by egg diameter and the differences between control and nucleotide larvae were subsequently found to be significantly different (F = 42.05, p = 0.000 and T = - 4.95, p = 0.000 for length and myotome height respectively) and higher for the nucleotide group. Larval growth rate was also significantly higher for the nucleotide larvae (T = - 4.79, p = 0.003; Table 4. 5; Figure 4. 15). No significant differences were found in the dry weight nor in the condition of the larvae at this stage (Table 4. 5).

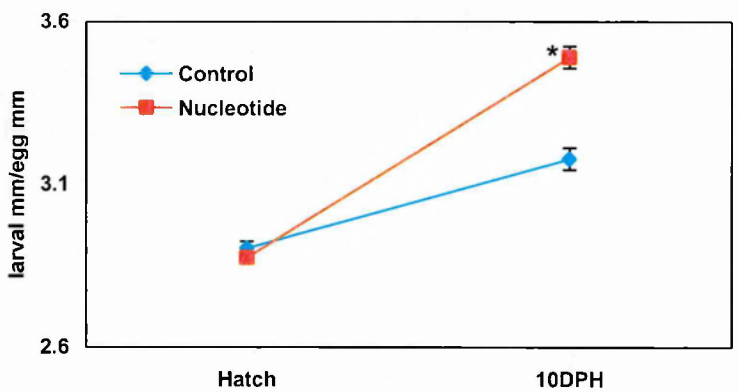


Figure 4. 15 Haddock larval growth from hatch until 10 DPH in 2003. Larval size is standardised with regard to egg size. Significant differences between diets are denoted with *. Mean ± SEM.

4.4.2.2 Larval performance

4.4.2.2.1 Survival

Larval survivorship was assessed five days after hatching in 2002 and 10 days after hatching in 2003. At the end of the first spawning season larval survival was checked at 42.5 °days after hatching and 82.59 ± 2.04 % of the stocked larvae from the broodstock fed the control diet

survived, compared to the 82.72 ± 2.68 % for the larvae from the nucleotide group. No significant difference was found between diets (F = 0.00; p = 0.970; Figure 4. 16).

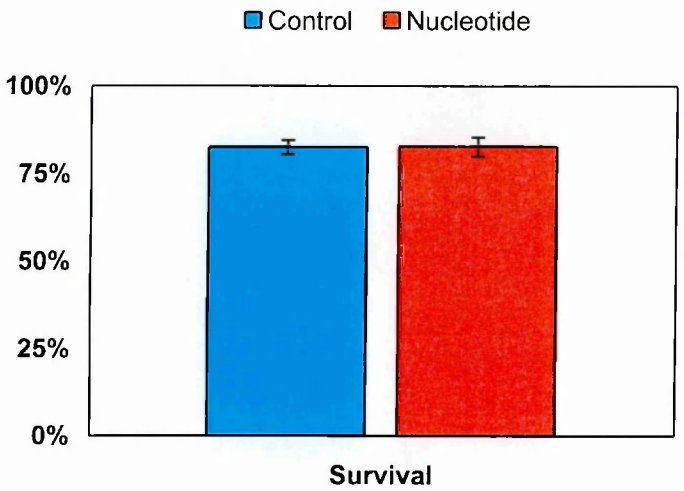


Figure 4. 16 Survival of haddock larvae at 5 days post hatching (DPH) in 2002 spawning season from the control (blue) and the nucleotide (red) diets. (Mean ± SEM).

In the second spawning season larval survival in the 18 vessels (3 vessels per batch, 3 batches per diet) was checked at 10 DPH (85 °days after hatching). At this time, live larvae were present in all the vessels and survival ranged from 31 to 88 % in the nucleotide group and from 3 to 72 % in the control vessels, with a mean survival of 60.91 ± 6.95 % for the nucleotide larvae and 40.43 ± 9.64 % for the controls. However, a significant difference could not be assured with 95% confidence but with 90 % instead (F = 2.97, p = 0.10; Figure 4. 17).

4.4.2.2.2 Gut development

Gut development studies in haddock larvae were carried out in 2003. Analysis showed clear differences in the developmental stage of the guts. After the 10-day experiment, 53 ± 14 % of the control larvae examined had advanced gut development, compared to 94 ± 6 % in the

nucleotide group (χ^2 = 28.6, p = 0.000; Figure 4. 18). From the remaining control larvae, 20% had poorly developed guts after 10 DPH (Figure 4. 1A).

4.4.2.2.3 First feeding success

Nucleotide larvae had a significantly greater (F = 5.46, p = 0.033) first feeding success; 51.21 ± 7.32 % versus 28.51 ± 6.39 % for nucleotide and control larvae respectively (Figure 4. 17). When carrying out an ANCOVA analysis, none of the batch or egg characteristics tested produced a significant effect when included in their respective models. From the larval variables, only the inclusion of gut development influenced significantly the first feeding success (Table 4. 8). This indicates that first feeding success of the NT larvae was significantly better as a result of enhanced gut development. First feeding success was also highly correlated with larval survival (r = 0.746, p < 0.001).

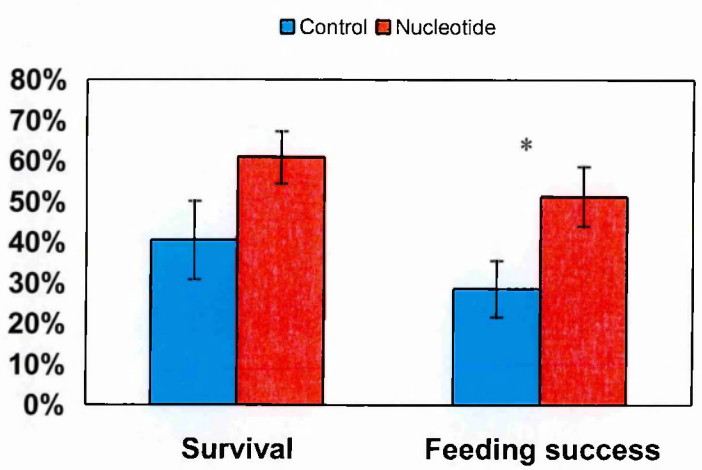


Figure 4. 17 Survival and first feeding success of haddock larvae at 10 DPH in 2003. Significant difference is denoted by *. (Mean ± SEM).

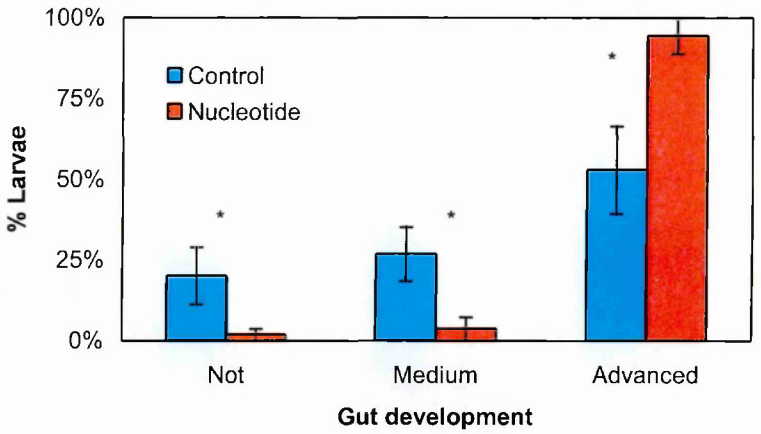


Figure 4. 18 Gut development of haddock larvae at 10 days post hatching (DPH). Significant differences denoted by *. Mean ± SEM.

Table 4. 8 ANCOVA analysis results on first feeding success of haddock larvae from broodstock fed two different diets (control and nucleotide) for 2 consecutive years. ** denotes significant differences.

Variation Source	DF	Sums of Squares	Mean Squares	F Value	P Value	R^2	R^2 –adj
Total	3	0.3889	0.1296	6.38	0.008**	61.47	51.84
Diet	1	0.0832	0.0832	4.10	0.066		
Gut Development	1	0.2883	0.2883	14.19	0.003**		
Diet*Gut Development	1	0.0833	0.0833	4.10	0.066		
Error	12	0.2437	0.0203				

4.5 Discussion

Information on the effects of broodstock nutrition on fish reproduction is still limited, but it is even sparser when looking at its influences on larval quality and survival. In the present study it has been demonstrated that larval quality can be improved through supplementing halibut and haddock broodstock diets with nucleotides.

Halibut trial

Although halibut larvae from both diets were of similar size to that reported in previous studies (e.g. Haug, 1990), control and nucleotide larvae differed in size (Table 4. 1). Halibut fed the nucleotide-enhanced diet spawned smaller eggs which in turn resulted in smaller larvae, in

terms of length, although the volume of their yolk sacs was not significantly different at hatch. However, when correcting for egg size, larval size was the same at hatch between treatments and the condition of nucleotide larvae was not compromised by their size. Length growth rate over the 42-day incubation period was similar in the larvae from the control and the nucleotide groups (Table 4. 1). However, as shown in Figure 4. 5, when larval size was corrected by egg size and the growth compared between both dietary treatments, the larvae from the nucleotide group grew significantly faster than the control and were significantly larger at the middle and end of the endogenous feeding stage. This phenomenon is confirmed by the fact that although control larvae were significantly heavier than nucleotide larvae at hatch, the weight differences had disappeared at 126 and 252 °days after hatch. Furthermore, small larvae at hatch had a higher growth rate at 252 °days when compared to bigger larvae (Figure 4. 6).

Halibut larvae from the nucleotide group suffered significantly lower mortalities that those from the control group during the endogenous feeding stage. Various hypotheses could be suggested to explain the higher survival of nucleotide larvae throughout the yolk sac stage. Manipulation of the yolk composition can be achieved through the broodstock diet (e. g. Watanabe, 1985). The supplementation of nucleotides to the diets of the broodstock may have improved the nutritional quality of the yolk, with a concominant nutritional advantage over the control larvae and hence better endogenous feeding. The nucleotide-enhanced diet may have 1) increased the levels of nucleotides in the eggs (this issue will be addressed in chapter 5) and/or 2) increased the concentrations of other nutritional components whose anabolic and/or catabolic metabolism is affected by nucleotides. Nucleotides are known to affect lipid metabolism in humans and other mammals (reviewed Carver & Walker, 1995). Ramirez *et al.* (1991) suggested that dietary nucleotides might modulate PUFA conversion and eicosanoid synthesis, since increased levels of arachidonic acid (20:4n-6), prostacyclin (PGI2) and thromboxane (TXA2) were found in the plasma of rats fed a nucleotide supplemented diet. Jimenez *et al.* (1992) showed that dietary nucleotides produced an increase in the n –6 long chain PUFA, especially 20:4n-6 (ARA) in the membrane of red blood cells of rats. Boza *et al.* (1992), studying plasma fatty acid levels in

weanling rats, also reported a significant increase in fatty acids, namely monounsaturated and poly-unsaturated fatty acids of the n – 3 and n – 6 series in the group fed the nucleotide diet, suggesting an important modulation of PUFA synthesis by dietary nucleotides. In a study looking at the effects of dietary nucleotides on lipid metabolism and the learning ability of rats it was reported that docosahexaneoic acid (22:6n-3) and arachidonic acid (20:4n-6) levels in the phosphatidylcholine fraction of the cerebral cortex were increased in the group fed the nucleotide supplemented diet (Sato *et al.*, 1995). Furthermore, the administration of a nucleotide-supplemented diet to rats corrected the altered levels of saturated, monounsaturated and n – 6 long-chain polyunsaturated fatty acids in plasma and liver microsomes produced by induced liver cirrhosis (Fontana *et al.*, 1998). Therefore, nucleotides included in the broodstock diet may have also altered the levels of the different PUFA in the broodfish but through what mechanism?

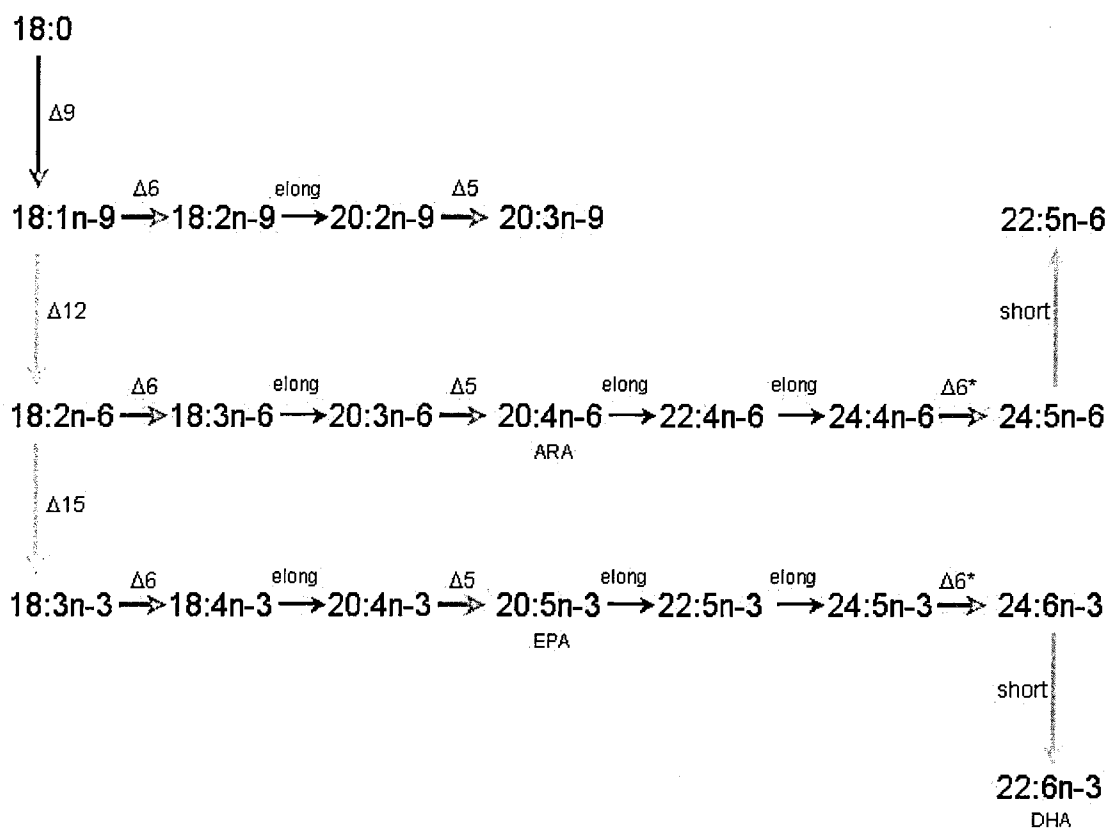


Figure 4. 19 Biosynthetic pathway of C_{20} and C_{22} HUFA from n – 3, n – 6, and n – 9 C_{18} precursors. Δ5, Δ6, Δ6*, Δ9, Δ12 and Δ15 are fatty acyl desaturases; elong: fatty acyl elongases; short: chain shortening. Δ12 and Δ15 are only found in plants. The Δ6* may or may not be the same enzyme (Δ6) that acts on C18 fatty acids. (Tocher, 2003).

Nuñez *et al.* (1993) suggested this ability of dietary nucleotides modifying the polyunsaturated fatty acid metabolism as a consequence of affecting activities of the desaturase enzymes involved in the PUFA biosynthetic pathways in rat liver; Δ_9-desaturase activity was lowered while Δ_5- and Δ_4- were increased. The importance of lipids and in particular HUFA during the early life-history of marine fish has been demonstrated throughout the literature (reviewed by Rainuzzo *et al.*, 1997; Sargent *et al.*, 1999; Bell *et al.*, 2003). However, there is evidence that the syntheses of EPA and DHA from linolenic acid (18:3n-3) and ARA from linoleic acid (18:2n-6) occur poorly if at all in marine fish species (Sargent *et al.*, 1989) and for this reason EPA, DHA and ARA are considered essential fatty acids (EFA) and have to be included in the diet of marine fish (Sargent *et al.*, 1999). There is no evidence at all of the existence of Δ_4-desaturase in fish (Tocher *pers. comm.*); however the Δ_5-desaturase is responsible for the production of ARA and EPA from 20:3n-6 and 20:4n-3 respectively (Figure 4. 19) and the limited activity of this enzyme was also suggested as responsible for the limited conversion of C18 to C20 PUFA in marine fish (Tocher & Sargent, 1990). In fact, Δ_5-desaturase activity has been cloned from zebrafish (*Danio rerio*) as part of a bifunctional Δ_5/Δ_6 activity (Hastings *et al.*, 2001) and also from salmon (Tocher, *pers. comm.*). Based on this, it could be suggested that as in rats, dietary nucleotides could stimulate the Δ_5-desaturase of broodfish and increase the production of ARA, EPA and then also DHA (formed from EPA) and therefore increase the content of these essential fatty acids in the eggs and improve the survival of the larvae from the nucleotide group. However, recent studies have demonstrated that the Δ_5-desaturase is not the only enzymatic activity responsible for the inability of marine fish to convert C_{18} PUFA precursors to C_{22} PUFA products. *In vitro* studies using cell lines have demonstrated that in turbot despite Δ_5-desaturase activity being high, the formation of EPA was limited by the low activity of the C_{18} to C_{20} elongase enzyme (Ghioni *et al.*, 1999). In contrast, gilthead seabream cell lines had active C_{18} to C_{20} and C_{20} to C_{22} elongase activities, but had very low Δ_5-desaturase activity (Tocher & Ghioni, 1999). The apparent inability of marine fish to convert 18:3n-3 to EPA and DHA is not due to the complete absence of the required genes in a particular species (in fact the genes are present) but due to the fact that they are not sufficiently well expressed

(C_{18} to C_{20} elongase in turbot and Δ_5-desaturase in gilthead seabream). Tocher (2003) stated that '*the problem may be how to switch on the recalcitrant genes*'. Furthermore, the function of the enzyme Δ_6-desaturase, present actively in all the marine fish species studied, is unknown (Tocher, 2003) and dietary nucleotides have also been suggested to affect its activity (Gil *et al.*, 1988; Pita *et al.*, 1988 cited by Fontana *et al.*, 1998). It is not clear whether or not the Δ_6* enzyme acting on C_{24} fatty acids is the same enzyme (Δ_6) that acts on C_{18} fatty acids (Figure 4. 19); therefore it can not be excluded that dietary nucleotides had affected the Δ_6* step and consequently the conversion of EPA to DHA. Manipulation of content of n – 3 and n – 6 HUFA in eggs has been successfully achieved through broodstock nutrition in different marine fish species causing an improvement of the larval quality (Fernández-Palacios *et al.*, 1995; Tandler *et al.*, 1995b; Furuita *et al.*, 2000; 2003; Mazorra *et al.*, 2003). Currently, no studies have been published regarding the elongase/desaturase activities in Atlantic halibut or haddock. However based on the extensive literature reporting the effects of nucleotides on the fatty acid metabolism of mammals, the use of dietary nucleotides as potential stimulants of the fatty acid desaturase or even elongase activities of marine fish should be investigated with both *in vitro* and *in vivo* models.

Nevertheless, this hypothesis of nucleotide-enhanced broodstock diets improving the nutritional quality of the yolk is also supported by the yolk absorption data. The fact that the yolk absorption rates were similar in both dietary groups may suggest that the enhanced growth of the nucleotide halibut larvae throughout the yolk-sac stage was a consequence of different yolk composition in the control and the nucleotide treatments.

Another possible hypothesis involves the development of the immune system in halibut larvae. Bowden *et al.* (2004) have described the ontogeny of some immunological parameters in halibut larvae. Lysozyme and complement C3b were present from the eggs onwards and their expression increased from hatch, whereas immunoglobulin M (IgM) was also present in eggs, IgM mRNA expression commenced around 20-25 DPH and the expression of the RAG1

(recombination activating gene) – essential for the normal functioning of T and B-lymphocytes – started about 10 days post hatch. Bowden *et al.* (2004) suggested that halibut larvae are immunocompetent fairly early in their development (around 10 DPH) and that the maternal contribution (lysozyme, C3b and IgM) would allow autologous development of the immune system without loss of protection. Nucleotides are well known to stimulate both the non- and the specific immune responses in fish (§ 1.4.4.5). As immunostimulants the nucleotide-enriched broodstock diet might have: 1) induced higher levels of nucleotide in the eggs which might have stimulated the development of the immune system in the larvae; and/or 2) immunostimulated broodfish increasing the maternal contribution of immunological-active compounds inside the eggs, and thereby increasing the immunocompetence and protection of the larvae during the yolk-sac stage. Although this mechanism has been suggested in various reviews (e. g. Vadstein, 1997), no studies have reported any attempts to stimulate the immune defences of larvae through the immunostimulation of the parents. However, a few studies showed that immunisation of female tilapia broodfish enhanced the maternal immunity of broodfish and increased larval resistance (Mor & Avtalion, 1990; Sin *et al.*, 1994). The survival of halibut larvae in the present study was assessed at 252 °days, (i.e. 42 DPH at 6 °C), when the larvae were immunocompetent. Halibut larvae have a long yolk sac stage during which they are exposed, in culture, to higher numbers of opportunistic pathogenic bacteria levels compared to what they experience in their natural condition (Hansen, 1993). Higher survival of nucleotide larvae may therefore have been a result of better immunological condition.

However, and most probable, a combination of both a higher nutritional value of the yolk during the endogenous feeding phase and an improved immunological condition may have resulted in a higher survival of the nucleotide halibut larvae.

Haddock trials

The size of the haddock larvae at hatch, in terms of length and myotome height, did not differ between the control and the nucleotide groups, even when corrected by egg size, in the 2002 and 2003 spawning seasons. Larval size (~ 4 mm) was similar to previous records reported by

Downing (2002). Yolk sac volume was also similar in 2002 between both dietary treatments but significantly different in 2003, the second spawning. Yolk sac volume of larvae from the nucleotide group were bigger than expected when corrected for egg volume in 2003. A reduction of the yolk sac in the control group is evident when data from both spawning seasons are compared. It may also be suggested that yolk-sac in control larvae was abnormally small, rather than that of nucleotide larvae being significantly bigger. The cause of this phenomenon is unknown and is more intriguing when the fact that egg size increased in both diets from 2002 to 2003 is considered (Chapter 3). However, this is not an isolated event since slight decreases in the mean length and myotome height over the two spawning seasons can also be observed in both dietary groups (Table 4. 5). Similarly, Clemmensen *et al.* (2003) reported that cod larvae from newly recruited spawners were significantly longer at hatch than those from repeat spawners.

Larval dry weight at hatch was not significantly different between diets in either season although there was a considerable increase from 2002 to 2003. However, this increase in the control larvae was not corresponded with an increase in their length or myotome height. It is therefore assume that water content in the 2002 larvae was higher than in 2003. Hislop (1988) demonstrated that haddock egg dry weight increases with the age of the broodfish, as also reported in the present study; therefore this could be the cause of the annual increase in the larval weight. Larval weights at hatch were slightly higher than those previously reported by Castell *et al.* (2003) for larvae of the same age. The different age of the broodstock could be the reason for this, however this sort of information is not available in Castell *et al.*'s paper. Larval weight gain over the yolk-sac stage was higher in the nucleotide group, while control larvae hardly increased their weight in those 5 days (Figure 4. 12). Reports of weight gain associated with nucleotide supplementation have also been described in small mammals (Ogita *et al.*, 2002), humans (Cosgrove *et al.*, 1996) as well as fish. Weights of vaccinated Atlantic salmon challenged with *Aeromonas salmonicida* were higher than control fish after 8 weeks of feeding on a nucleotide-enhanced diet (Burrells *et al.*, 2001b). Growth of gilthead seabream was also improved when larvae (Borda *et al.*, 2003), juveniles (Oliva-Teles *et al.*, 2003) and adults (A.

Fernández-Vaquero *pers. comm.*) were fed nucleotide diets. Juveniles of hybrid striped bass (*Morone chrysops* X *M. saxatilis*) fed a nucleotide-boosted diet and exposed to *Streptococcus iniae* tended to have greater weight gain (Li *et al.*, 2004). In all these studies the fish were directly fed the nucleotide diet. However this is the first study that reports a nucleotide effect on the growth of yolk-sac larvae through broodstock diet supplementation. As with halibut, this could be a consequence of nucleotide-enriched diets improving the quality of the yolk, used as the only nutritional source during the endogenous feeding stage (already discussed above).

Survival of haddock larvae during the yolk-sac stage was similar in both dietary treatments and above 80% indicating that the utilisation of glass bowls is a feasible culture technique to follow haddock larval development and survival through the yolk-sac stage, as for halibut (Lein & Holmefjord, 1992). However, this system was not suitable to maintain the haddock larvae alive for longer periods and study their first feeding success. Therefore plastic vessels were used during the second spawning season.

As mentioned above, in 2003, larvae from haddock broodfish fed a nucleotide-enhanced diet were not significantly bigger at hatch but had a significantly larger yolk sac, better gut development rate, growth rate, first-feeding success and larval survival at 10 DPH. Differences in the yolk-sac volumes between diets were a consequence of the nucleotide group producing significantly bigger eggs (Table 3.7). According to Hamlin *et al.* (2000), the digestive tract of haddock larvae appears as a simple straight tube, dorsal to the yolk-sac at hatch and 30-36 h later the mouth opens and exogenous first feeding begins. A single ventral convolution toward the posterior end of the intestine and a constriction separates the mid-intestine from the hindgut by day 2 – 3 with the intestine becoming much wider anteriorly and increasing in size thereafter. The development of pyloric caeca, which is the last major morphological change of intestinal development, takes place by day 35 after hatch; equivalent to 10 mm body length (Hamlin *et al.*, 2000). This was far beyond the duration of our experiment and therefore the guts were not fully developed at 10 DPH. The guts of the nucleotide larvae however, were significantly more developed than the control larvae ten days after hatch. Burrells *et al.* (2001b) reported similar

effects after three weeks of feeding adult Atlantic salmon with a nucleotide enhanced diet and histological observations revealed that the total gut surface area was bigger due to increased lateral branching of the intestinal folds.

There is an extensive literature reporting the importance of nucleotides on the gastrointestinal tract in mammalian studies, both *in vitro* and *in vivo*. Dietary nucleotides are important for nucleic acid synthesis in tissues with a high cell turnover (such as the gastrointestinal tract) and since gut epithelial cells have a limited capacity for de novo nucleotide synthesis (Leleiko *et al.*, 1983), nucleosides and nucleotides are considered semi-essential nutrients for optimal gut growth and development (Uauy *et al.*, 1990). Nucleotides increase the growth and differentiation of the intestine in rats and infants (Uauy *et al.*, 1990, 1994; Carver *et al.*, 1993; Tsujinaka *et al.*, 1993; Carver, 1994). Different studies have found that their deprivation is detrimental for intestinal maturation (Ortega *et al.*, 1995b) and protein synthesis and DNA concentration (López-Navarro *et al.*, 1996b). Furthermore, all these findings from *in vivo* experiments have been complemented with those from *in vitro* ones. A few studies have confirmed that nucleotide supplementation also enhances growth and maturation of rat and human intestinal cell lines when nutrient supply is limited (He *et al.*, 1993; Holen & Jonsson, 2004).

The importance of successful first feeding to larval survival is well established in all cultured species. The transition from endogenous to exogenous feeding of young larvae is a critical step in larval cultivation and is usually associated with high mortalities. It is suggested that the nucleotide supplementation in broodstock feed led to an improved and accelerated gut development in the larvae, which could then start feeding on rotifers earlier. Thus, in the present study the enhancement of the intestinal development of larvae in the nucleotide group led to a significantly higher feeding success (Figure 4. 17); in fact, gut development and feeding success were positively correlated.

Feeding success was also positively correlated with larval growth, despite control and nucleotide larvae having the same length at hatch. Improvement in growth of rainbow trout has been reported by Rumsey *et al.* (1992) and Adamek *et al.* (1996) when increasing the content of nucleotides in the diet. Burrells *et al.* (2001b) described that the typical growth depressions seen

in fish after vaccination were not observed in fish fed a nucleotide-enhanced diet. Recently, Peres and Oliva-Teles (2003) reported that growth levels of juvenile seabass decreased when using yeast-RNA extract at high levels (12% diet). However, dietary RNA was used as a nutrient replacement in low protein diets and not as an additive as nucleotides were in this study. Oliva-Teles *et al.* (2003) confirmed that high RNA levels depress growth performances, possibly due to an insufficient capacity in juvenile gilthead seabream to degrade nucleic acid into urea by uricase. However, in the present study, the improved growth rate was probably a result of the better gut development in the nucleotide larvae, allowing them to start first feeding earlier than the control group, which in turn was possibly due to a nutritionally-improved yolk.

Chapter 5: Adenine nucleotide content and energy charge

5.1 Introduction

The adenine nucleotides (ATP, ADP, AMP) are ubiquitous in all living cells, and are often referred to as energy storage compounds (e. g. Boulekbache, 1981). However, on a calorific basis they contain a relatively small amount of energy when compared to lipids or carbohydrates. Instead, the balance of the concentrations of these nucleotides has a major role in the regulation of catabolic and anabolic metabolism (Atkinson, 1968). Thus, high concentrations of ATP relative to ADP and AMP inhibits glycolysis and stimulates gluconeogenesis and presumably growth. However, when levels of ATP are low, compared to ADP and AMP, glycogenolysis and ATP regeneration is stimulated through glycolysis and the tricarboxylic acid cycle.

The concept of energy charge (EC) was first adopted by Atkinson & Walton (1967) as a measure of the energy directly available to cells:

$$EC = \frac{[ATP] + 0.5 \times [ADP]}{[ATP] + [ADP] + [AMP]} \qquad \text{[Eq.5. 1]}$$

The EC is the fraction of usable high energy bonds in the total adenine nucleotide pool. The numerator includes ADP because of the activity of adenylate kinase in cells:

$$ATP + AMP \leftrightarrows 2\ ADP$$

The reaction has an equilibrium constant near one; thus two ADP molecules can be used to make one ATP molecule. The 0.5 in the equation normalises the value between 0 (all AMP) and 1 (all ATP) (Atkinson, 1968). Since ATP, ADP and AMP are ubiquitous molecules, EC can be measured in any organism. Ivanovici (1980) also suggested this ratio as an ecophysiological index of the 'well-being' of organisms. Thus, high values (between 0.8-0.9) have generally been found in organisms which are in a non-limiting environment, and which are growing and reproducing. Values between 0.5 and 0.7 have been found in organisms whose environment is limited in some way (e.g., lack of nutrients, oxygen). Such organisms have reduced growth rates and may not reproduce. Energy charge values below 0.55 are typical of organisms under severe stress conditions and might compromise viability even when returned to optimal conditions.

Adenylate EC ratio has been used as an indicator of fish condition in several studies. For instance, Vetter & Hodson (1982) demonstrated that the use of EC measured in white muscle is a valid indicator of hypoxic stress in 11 estuarine fish species. Toxicological studies are also known to use this ratio to report the condition of fish; pesticides were reported to decrease the EC of gill tissue in bluegill sunfish (*Lepomis macrochirus*) (Hohreiter *et al.*, 1991).

ATP and/or EC levels in eggs from marine and freshwater fish species have been measured in a few studies (Vetter *et al.*, 1983; Boulekbache *et al.*, 1989; Wendling *et al.*, 2000, 2004; Lahnsteiner & Patarnello, 2003; Aegerter & Jalabert, 2004). Vetter *et al.* (1983) reported a decline in EC ratio from 0.87 after fertilisation to 0.58 prior to hatching in red drum (*Sciaenops ocellata*). Furthermore, Bouleckbache *et al.* (1989) reported that the ageing of fish oocytes was associated with a depletion of ATP levels which induced a continuous decrease in the EC correlated with the ageing process. The survival of carp embryos was reported to be minimal when EC reached its lowest value. Investigations on viable and non-viable seabream eggs failed to demonstrate a significant difference in ATP levels, but revealed a much higher variability in the non-floating egg fraction and the EC ratio of non-viable eggs decreased during development while viable eggs maintained a constant EC (Lahnsteiner & Patarnello, 2003). Furthermore, depletion of ATP content and EC associated with reduced milt motility during the spawning

season have been described in salmonids (Benau & Terner, 1980) and seabass (Dreanno *et al.*, 1999). More recently Zilli *et al.* (2004) demonstrated the use of ATP concentration as an indicator of milt quality in seabass. Based on these facts, variation in adenine nucleotides and EC in halibut and haddock egg and larval samples were studied during the spawning season in the present project.

5.2 Aims

The aims of this part of the project were:

- To establish whether the nucleotide-enhanced broodstock diet affected the adenine nucleotide content and energy charge of eggs and larvae from halibut and haddock broodstock.
- To study the variation of the different adenine nucleotides and energy charge during the spawning season.
- To investigate if the provision of extra nucleotides in the broodstock feed caused an increase in the content of these compounds in halibut and haddock progeny.

5.3 Material and Methods

Analysis of adenine nucleotide content can be carried out by two techniques: luminescence or chromatography. The luminescence (the emission of light produced by a non-thermal process) technique is based on the activity of the luciferin-luciferase enzyme. Thus, the amount of light produced in a chemical reaction between ATP and a fluorescent compound called luciferin is catalysed by the luciferase enzyme in the presence of oxygen and a divalent cation (e.g. Mg^{2+}, Mn^{2+}) (Lundin, 1984). In this technique the concentration of ATP is proportional to the light emitted which can be quantified with a photometer. The levels of ADP and AMP can also be measured by following steps through the enzymatic transformation of these compounds to ATP.

The development of high performance liquid chromatography (HPLC) in the last 20 years has led to an increase in literature on biomolecule separation. HPLC seems to offer several advantages when compared to the luciferin-luciferase method: in a single run, several nucleotides, in particular ATP, ADP, AMP, can be directly separated and quantified at very low concentrations. Furthermore, chloride salts (present in marine organisms) that cause inhibition of the luciferase assay (Pradet, 1967) do not have any adverse effect on HPLC separation (reverse phase type). Therefore, in the present study, the adenine nucleotide content of egg and larval samples was determined using HPLC methodology. Chromatography is a separation technique involving mass-transfer between stationary and mobile phases. HPLC utilises a liquid mobile phase to separate the components of a mixture (nucleotides of egg and larval samples in this case). These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under high pressure. In the column, the mixture is then resolved into its components which are eluted at different times. The output is a chromatogram with a series of peaks which correspond to the different compounds included in the mixture. The concentration of each compound is then calculated by integrating the area under each peak.

5.3.1 Sample collection

Egg and larval samples from halibut and haddock broodstock were collected at different stages of their development in the 2001 and 2002-2003 spawning seasons respectively. Halibut eggs were stripped from females so egg samples were collected prior to and after fertilisation, and larval samples at hatch, 126 °days and 252 °days from all the incubated batches. Whereas in haddock, eggs were already fertilised when collected, so egg samples were taken at the morula stage and larval samples at hatch and 5 days post hatching (DPH) in 2002 and only at hatch in 2003 (Table 5. 1)

Table 5. 1 Stages at which samples were taken for nucleotide content analyses during the project.

Species (Year)	Egg samples	Larval samples
Halibut (2001)	Prefertilised and postfertilised	Hatch, 126 °days and 252 °days
Haddock (2002)	Postfertilised	Hatch and 5 DPH
Haddock (2003)	Postfertilised	Hatch

Rapid freezing is critical for the accurate determination of labile compounds such as ATP, ADP, and AMP (Faupel *et al.*, 1972). Therefore, once collected, egg and larval samples were rapidly weighed and immediately stored in liquid nitrogen at -196 °C until the biochemical analyses were carried out.

5.3.2 Sample analysis

Two types of analysis were carried out using halibut and haddock egg and larval samples:

- Quantification of adenine nucleotides (i.e. AMP, ADP, ATP) (§ 5.3.2.1).
- Quantification of nucleotide content – total potentially available nucleotides (§ 5.3.2.2).

Adenine nucleotide content and nucleotide analysis were carried out using two different chromatographic systems and sample preparation methods, which are described bellow.

5.3.2.1 Adenine nucleotide content

5.3.2.1.1 Sample preparation

The preparation of samples and nucleotide extraction was adapted from Bouleckbache *et al* (1989). Frozen egg and larval samples were transferred to liquid-nitrogen precooled centrifuge tubes (50 ml capacity). Proteins and therefore also enzymes were denaturised by adding 4 ml of

precooled 10 % perchloric acid (PCA) 1mM EDTA solution and eggs and larvae crushed using a glass rod (also precooled in liquid nitrogen). The mixture was left in ice for 15 min, until samples were centrifuged for 10 min, at 2 °C and 5,000 rpm. The supernatant was collected and the pellet resuspended and extracted with PCA-EDTA solution again for 15 min. Samples were centrifuged again for 10 min, at 2 °C and 5,000 rpm and both supernatants containing the nucleotides were combined. Supernatants were added to 0.04 % phenol red solution (volume 1% of sample volume) in order to follow neutralisation with ice-cold potassium hydrogen carbonate ($KHCO_3$) 5M solution. The mixture was centrifuged at 2 °C and 5,000 rpm, the supernatant carefully pippeted out into ice-cold 13-ml centrifuge tubes, filtered using 13 mm diameter 0.2 μm pore size disposable PTFE syringe filters (Whatman®) and injected into the HPLC.

5.3.2.1.2 Chromatographic conditions

Levels of adenine nucleotides (AN) – ATP, ADP and AMP – were quantified using a Beckman System Gold™ HPLC, composed of a 126 Solvent Module, a 507e Autosampler and a 166 Detector (UV) set up at 254 nm wavelength. The HPLC column used was a reverse-phase C-18 Lunar 150 x 4.2 mm (length x internal diameter), from Phenomenex (U.K.).

Mobile phase buffer A was a 0.1M ammonium dihydrogen phosphate ($NH_4H_2PO_4$) solution at pH = 6.0; while mobile phase buffer B was methanol (HPLC grade) diluted 50 % with 0.2 M $NH_4H_2PO_4$ solution with a pH of 6.0. The extracted sample was injected (30 μl) and mobile phase A ran at 100 % for 10 min. (isocratic flow) followed by a linear decline to 0 % with a concurrent increase in buffer B to 100 % over a 20 min. period (gradient flow). After this, buffer A was returned to 100 % and buffer B to 0 % in the following 5 min (isocratic flow). Flow rate was at 1 ml / min and a single run was terminated after 40 min.

Apart from ATP, ADP, and AMP, other compounds such as IMP, inosine (Ino), hypoxanthine (Hyp), xanthine (Xan) and uric acid (UA) were also measured. Since these last five compounds

are obtained by degradation of AMP, the quantification of them was used to reject any sample in which adenine nucleotides had degraded possibly due to thawing. Under the above chromatographic conditions, elution times were approximately as follows (Table 5. 2):

Table 5. 2 Elution times and standard concentrations of the adenine nucleotides and related compounds quantified by HPLC analysis.

Compound	Elution time	Standard concentration
Uric acid	3.5 min	25.0 mM
IMP	5.4 min	19.1 mM
ATP	6.2 min	280.9 mM
ADP	7.3 min	26.7 mM
Hypoxanthine	8.1 min	24 mM
Xanthine	9.8 min	24 mM
AMP	12.5 min	8.5 mM
Inosine	18 min	24 mM

Standard solutions containing the above eight compounds were prepared (Table 5. 2) and a series of dilutions were injected to produce standard curves and calibrate the system. Daily injections of standards to confirm calibration were also performed. The most diluted solutions (lowest concentration) were injected first in order to prevent over-loading of the HPLC column between injections.

The concentration of each adenine nucleotide was initially expressed as μmol/l, but since egg and larval dry weights had been measured the concentration of each adenine nucleotide was finally expressed as μmol per gram of dry weight. Energy charge ratios were calculated for each sample according to:

$$EC = \frac{[ATP] + 0.5 \times [ADP]}{[ATP] + [ADP] + [AMP]}$$

5.3.2.2 Nucleotide content

Orally taken nucleotides administered via the diet are hydrolysed to nucleosides by alkaline phosphatases and nucleotidases. Nucleosides may be further broken down by nucleosidases to purine and pyrimidine bases, however it seems nucleosides are the primary form absorbed into the enterocytes (reviewed by Carver & Walker, 1995). Leach *et al.* (1995) developed a procedure to measure the Total Potentially Available Nucleoside Content in human milk, the TPAN method. The total potentially available nucleotide fraction contains nucleic acids, monomeric nucleotides, monomeric nucleosides and nucleoside-containing compounds such as NAD^+, UDP-glucose among others. The TPAN method is a bioanalytical approach to the measurement of the nucleotide content of a biological sample. It couples simulated *in vivo* enzymatic digestion of the various forms of nucleotides to the corresponding nucleoside with HPLC determination of the free and enzymatically-liberated nucleosides (Figure 5. 1). This method was adapted for the analysis of halibut and haddock egg and larval samples in the present study.

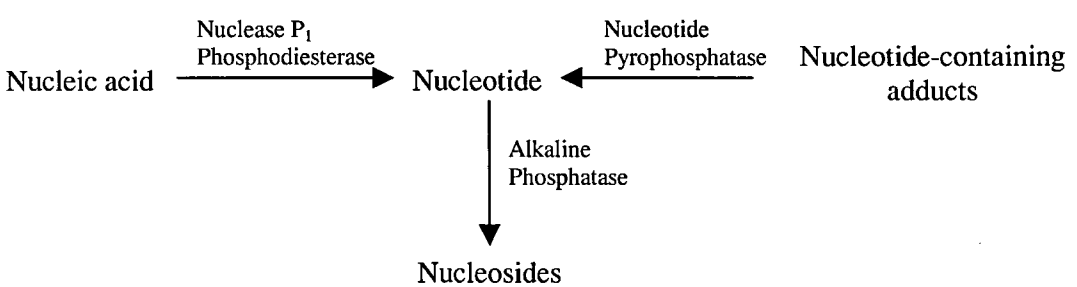


Figure 5. 1 Enzymatic digestion used in the preparation of samples for the TPAN method (Leach *et al.*, 1995).

5.3.2.2.1 Sample preparation

The preparation of the samples and the nucleotide extraction was adapted from Leach *et al.* (1995). Phosphorylated nucleotides were transformed into their corresponding nucleosides by enzymatic treatment of the samples as well as pH changes during sample purification. Frozen

egg and larval samples were transferred to 10ml Pierce Reacti-Vials, crushed with a precooled glass rod and 3 ml of 50 mM sodium acetate pH 5.1 buffer (Anal.R grade, Sigma), 50 µl of 10 mM zinc sulfate (Anal.R grade, Sigma), 50 µl of nuclease P1 enzyme solution (Sigma) and 50 µl of methyl cytidine (internal standard) (Sigma) added, then placed on a Reacti-Therm III Heating/Stirring block (Pierce) at 37 °C stirring slowly for 16-18 hours. Vials were removed and enzyme hydrolysis continued with a second step in which 50 µl of 30 % (w/w) ammonium hydroxide (Anal.R grade, Sigma), 1 ml of 0.5 M ammonium acetate pH 8.75 solution (Anal.R grade, Sigma), 50 µl of 1.0 M magnesium chloride (Anal.R grade, Sigma), 50 µl of phosphatase enzyme solution (Sigma) and 50 µl of pyrophosphatase enzyme solution (Sigma) were added. Vials were then returned to the heating/stirring block and allowed to incubate for 3 hours at 37 °C. The vial contents were transferred to 50 ml centrifuge tubes, neutralised with 2.5 ml of 0.5 sodium phosphate, pH = 10.5 (Anal.R grade, Sigma) and brought to volume using Milli-Q water. Contents were mixed thoroughly and centrifuged for 15 min at 3,000 rpm.

The enzymatically-treated sample was added into an ion-exchange solid phase extraction (SPE) column (Baxter Scientific) containing Affi-Gel 601 (Bio Rad). The nucleosides were bound to the matrix gel and all the unbound sample components were washed from the gel using 20 ml of 0.25 M sodium phosphate buffer, pH 10.5. Bound nucleosides were eluted into 10 ml centrifuge tubes by slowly passing 2 ml of 1.0 M phosphoric acid, followed by an additional 5 ml of 0.1 M phosphoric acid solution through the column. Purified samples were brought to volume with Milli-Q water, mixed and were then ready for injection into the HPLC.

Mixed nucleotide standard solutions containing AMP, CMP, GMP and UMP standards (Sigma), were also treated in the same way as the egg and larval samples.

5.3.2.2.2 Chromatographic conditions

All the phosphorylated nucleotides were enzymatically hydrolysed to their constituent nucleoside forms and analysed using HPLC. Levels of the different nucleosides (NS) (i.e.

adenosine, cytidine, guanosine, inosine and uridine) were measured on a Bio-Rad Biologic DuoFlow™ HPLC system with a UV detector at 254 nm wavelength. The HPLC column used was a Uptisphere 5 µm HDO, 250 x 4.0 mm (INTERCHIM, France).

Mobile phase buffer A consisted of a 0.05M potassium phosphate 2.5 % methanol (HPLC grade) solution at pH = 4.0 while mobile phase B was 0.05M potassium phosphate 20 % methanol (HPLC grade) and pH = 4.0. The extracted sample was injected (1 ml) and mobile phase A ran at 100 % for 6.25 min. (isocratic flow) followed by a linear decline to 0 % with a concurrent increase in buffer B to 100 % for 20 min. (gradient flow). After this buffer A was returned to 100 % and buffer B to 0 % in the following 3.75 min. and buffer A was run for 6.25 min to stabilise the system (isocratic flow). Flow rate was 0.8 ml / min and a single run terminated after 30 min.

Under the above chromatographic conditions, the elution times were approximately as follows:

Nucleoside	**Elution time**
Cytidine (Cyd)	7.9 min
Uridine (Urd)	11.3 min
Methyl-Cytidine*	12.3 min
Guanosine (Guo)	16.3 min
Adenosine (Ado)	20.5 min

(*) Internal standard

Standard solutions containing known amounts of the four mononucleotides and the internal standard (methyl-cytidine), which were treated similarly to the egg and larval samples, were injected before and after the injection of biological samples daily. Peak areas were measured and concentration of each nucleoside calculated according to the internal standard technique. This method is based on the addition of a known compound, called internal standard, at a fixed concentration to the unknown sample so the areas of the other compounds in the calibration curves are normalised by the area of the internal standard. The internal standard must a) resolve completely from the other peaks; b) elute close to the other nucleosides; c) behave similar to the other nucleosides; d) not be present in the original sample; e) be stable; unreactive with the sample components; and f) be of high purity (Hamilton & Sewell, 1982). Methyl-cytidine was

the internal standard used in the present study and the concentration of each compound of the mixture is calculated according to Eq. 5. 2:

$$NS\ conc. = \frac{Area\ NS\ (sample) / Area\ IStd\ (sample)}{Area\ NS\ (std) / Area\ IStd\ (std)} x\ Std\ conc. \qquad \textbf{[Eq.5. 2]}$$

where:

Area NS (sample): nucleoside area in the sample,

Area IStd (sample): area of the internal standard (methyl cytidine) in the sample,

Area NS (std): nucleoside area in the standard sample

Area IStd (std): area of the internal standard (methyl cytidine) in the standard sample

Std conc.: standard concentration of the nucleoside expressed as milligrams per litre (mg/l).

NS conc.: nucleoside concentration was initially expressed as mg/l, but since egg and larval dry weights had been measured NS concentration was finally expressed as μmol per gram of dry weight.

5.3.3 Data analysis

Statistical analysis of the data was as described previously (§ 2.3.6). Normality and homoscedasticity were studied for all the parameters. Box – Cox transformations were carried out to make non-normally distributed variables follow a normal distribution (Sokal & Rohlf, 1981). Parametric or non-parametric tests (ANOVA test, Man-Whitney test and Mood's tests) were then used to examine differences between both dietary treatments when appropriate. General linear models (GLM) as defined previously (§ 2.3.6) were also used to take into account all the parameters that could influence the response of the variable. ANCOVA tests were used to study the effect of the diet on a parameter (e.g. ATP content) that was suspected to be influenced by a covariate (e.g. date), the least squared means analysis was additionally used if the interaction term of diet-covariate (diet*covariate) was significant. Correlation and regression analyses were used to study relationships between parameters.

5.4 Results

5.4.1 Atlantic halibut

Adenine nucleotide contents were measured in halibut egg and larval samples from the control and nucleotide dietary groups, and EC calculated.

The content of ATP, ADP and AMP of halibut eggs– expressed as μmol per dry weight gram – prior to fertilisation did not differ between the control and the nucleotide diets ($T = 0.32$, $p = 0.753$; $F = 0.20$, $p = 0.660$; $F = 0.17$, $p = 0.687$ respectively) (Table 5. 3) and did not vary over season. Energy charge of prefertilised eggs was not significantly different between both diets at this stage (C: 0.76 ± 0.32; NT: 0.77 ± 0.02; $W = 128.0$, $p = 0.948$) and regression analysis did not show any seasonal pattern ($F = 0.00$, $p = 0.986$). After fertilisation, adenine nucleotide content continued to be similar between both dietary groups (ATP: $F = 1.26$, $p = 0.274$; ADP: $F = 0.62$, $p = 0.441$; AMP: $F = 0.67$, $p = 0.422$; Table 5. 3). ATP did not follow any seasonal trend; however, ADP was positively correlated with date ($r = 0.608$, $p = 0.001$; Figure 5. 2) and AMP content decreased along the spawning season ($r = - 0.423$, $p = 0.035$). The concentration of ATP in eggs from the control group fell after fertilisation, whereas it appeared to remain constant in eggs from the 'nucleotide-fed' broodstock. As a result of this, egg EC was significantly higher in the nucleotide group (C: 0.75 ± 0.03, NT: 0.82 ± 0.02, $F = 6.92$, $p = 0.017$; Table 5. 4) and increased after fertilisation (Table 5. 3).

Table 5. 3 Adenine nucleotide content in Atlantic halibut egg and larval samples. Expressed as µmol / g (dry weight). Significant differences are denoted by *. Mean ± SEM. Control: n = 11; Nucl.: n =11).

	ATP[1]		ADP[1]		AMP[1]		EC	
Stage	Control	Nucl.	Control	Nucl.	Control	Nucl.	Control	Nucl.
Prefertilisation	0.57 ± 0.05	0.55 ± 0.02	0.19 ± 0.02	0.17 ± 0.02	0.17 ± 0.09	0.11 ± 0.03	0.76 ± 0.03	0.77 ± 0.02
Postfertilisation	0.47 ± 0.04	0.54 ± 0.05	0.12 ± 0.02	0.1 ± 0.02	0.13 ± 0.04	0.08 ± 0.02	0.75 ± 0.03	0.82 ± 0.02 *
At Hatch	9.43 ± 2.96	11.39 ± 1.2	3.44 ± 1.21	2.78 ± 0.72	1.06 ± 0.44	0.87 ± 0.24	0.8 ± 0.02	0.86 ± 0.03
At 126 ° days	0.85 ± 0.34	0.64 ± 0.15	0.59 ± 0.2	0.69 ± 0.19	0.69 ± 0.26	0.29 ± 0.08	0.51 ± 0.11	0.61 ± 0.06
At 252 ° days	3.57 ± 2.95	2.29 ± 1.35	3.60 ± 1.4	1.84 ± 0.37	1.56 ± 0.35	1.02 ± 0.35	0.50 ± 0.08	0.55 ± 0.09

[1] : µmol / g (dry weight)

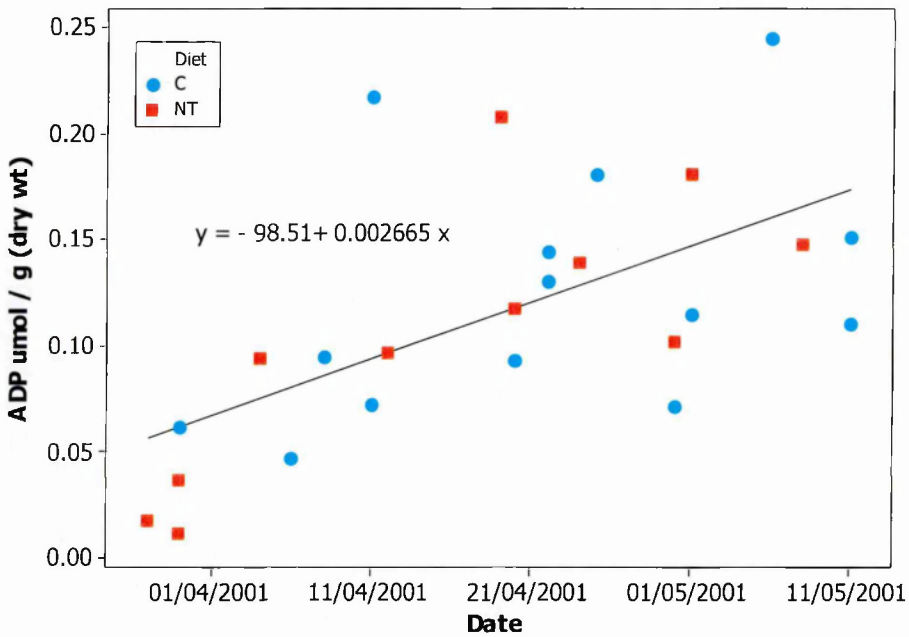


Figure 5. 2 Variation of ADP content in fertilised halibut eggs during the 2001 spawning season. Control (C): Blue circles, Nucleotide (NT): red squares.

Table 5. 4 GLM analysis of the energy charge (EC) of fertilised halibut eggs in the 2001 spawning season. Significant differences are denoted by *. R^2 = 40.26 %, R^2adj = 20.34 %.

Source	DF	Sums of Squares	Mean Squares	F Value	P Value
Diet	1	0.0587	0.0587	6.91	0.017*
Tank (Diet)	4	0.0361	0.0090	1.06	0.403
Date	1	0.0217	0.0217	2.56	0.217
Error	18	0.1527	0.0085		

No significant differences between diets were found in the ATP, ADP and AMP content of larval samples at hatch, 126 ° days and 252 ° days (Table 5. 3). Correlations of ATP, ADP and AMP with 'date' were not significant at any stage, although ADP and AMP contents in 252 °day-old larvae were almost positively and negatively correlated to date ($r = 0.58$, $p = 0.079$; $r = -0.59$, $p = 0.072$; Table 5. 5) respectively. Energy charge in larval samples was not significantly different between diets at any of the three stages sampled (hatch: $F = 2.43$, $p = 0.180$; 126 ° days: $F = 0.48$, $p = 0.519$; 252 ° days: $F = 0.20$, $p = 0.664$). When EC from both dietary treatments were pooled and the variation along the season studied, EC at hatch and 126 ° days were not correlated with date, indicating no seasonal trend ($p = 0.98$; $p = 0.543$ respectively). However, larval EC at 252 ° days significantly decreased along the spawning season ($r = -0.634$, $p = 0.049$; Figure 5. 3; Table 5. 5).

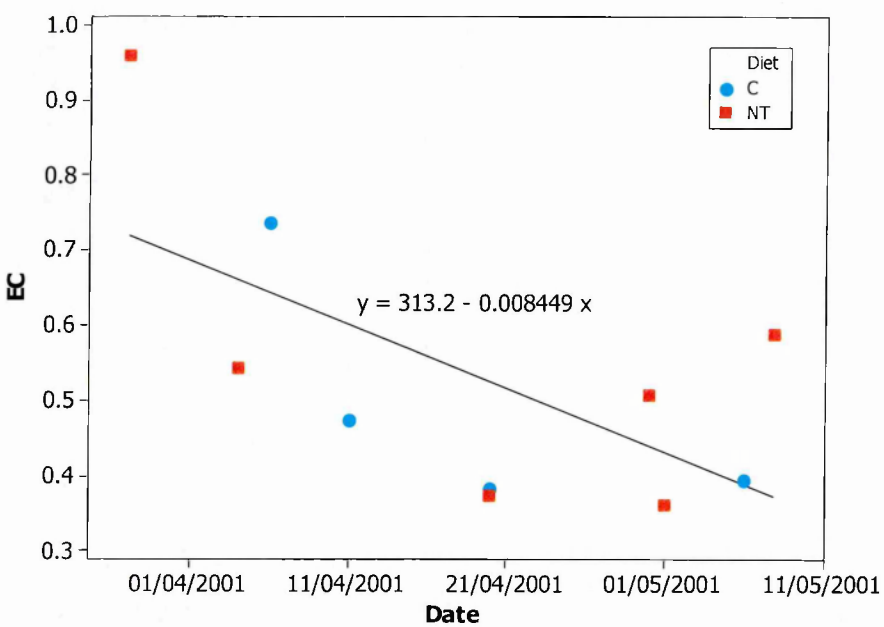


Figure 5. 3 Variation of energy charge (EC) in halibut larvae at 252 °days post hatch during 2001 spawning season. Control (C): Blue circles; Nucleotide (NT): red squares.

Control and nucleotide data were then pooled and variation of ATP, ADP, AMP and EC between egg and larval stages was studied (Figure 5. 4). ATP levels remained stable after fertilisation ($p = 0.999$) and increased at hatch ($p < 0.001$) followed by a decrease ($p < 0.001$) at

126 ° days to levels similar to those at the egg stage and finally increased slightly at 252 ° days compared to pre- and post- fertilisation stages ($p = 0.025$; $p = 0.017$ respectively) but not when compared to 126 ° days ($p = 0.206$). ADP followed the same pattern, although in this case ADP content in larval samples increased significantly from 126 ° days to 252 ° days ($p < 0.001$). AMP levels were maintained after fertilisation ($p = 0.993$) and increased at hatch ($p < 0.001$). However they appeared to remain stable thereafter when compared to hatch values (126 ° days: 0.585; 252 ° days: $p = 0.999$). Energy charge changes from egg to hatch were not significant ($p = 0.820$). However, EC ratio decreased at 126 and 252 ° days when compared to hatch values ($p < 0.001$; $p < 0.0010$ respectively).

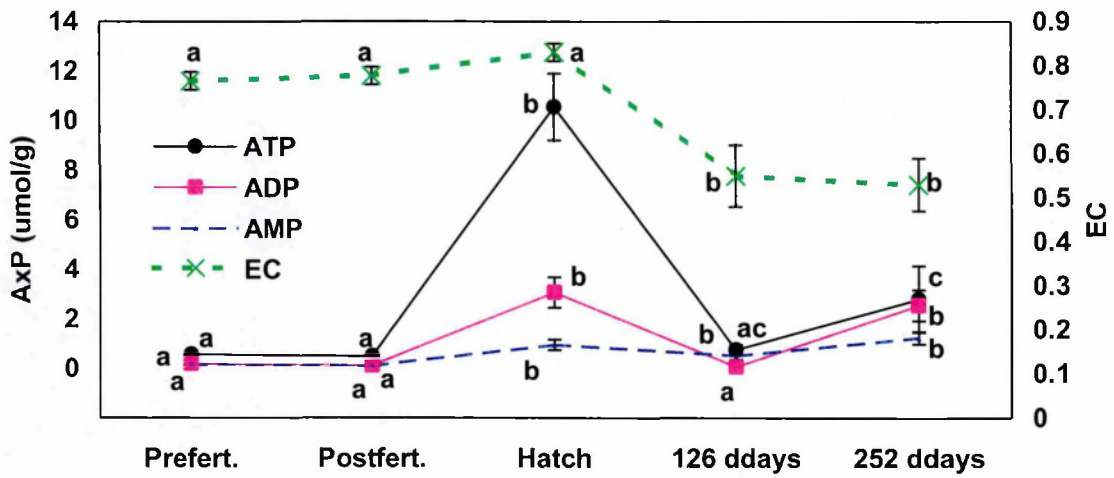


Figure 5. 4 Variation of adenine nucleotides and energy charge (EC) in Atlantic halibut eggs and larvae until the end of yolk resorption in the 2001 spawning season. Data points with different letters of the same colour indicate a significant difference. Mean ± SEM

Relations with other egg and larval parameters

Levels of ATP and EC in egg samples were also studied for possible correlations with fertilisation rate, hatching rates, blastomere morphology index and larval survival but all were not significant ($p > 0.05$). However, correlations between ATP levels in unfertilised and fertilised eggs and the drop-out 24h after fertilisation of halibut eggs were almost significant ($r = - 0.414$, $p = 0.099$; $r = - 0.434$, $p = 0.056$).

Table 5. 5 Correlations of adenine nucleotides (ATP, ADP & AMP) and energy charge (EC) in halibut egg and larval samples along the 2001 spawning season. Significant correlations are denoted in bold format.

Cell content: r - value / p - value		DATE	Prefertilisation ATP	Prefertilisation ADP	Prefertilisation AMP	Prefertilisation EC	Postfertilisation ATP	Postfertilisation ADP	Postfertilisation AMP	Postfertilisation EC	Hatch ATP	Hatch ADP	Hatch AMP	Hatch EC	126 ° days ATP	126 ° days ADP	126 ° days AMP	126 ° days EC	252 ° days ATP	252 ° days ADP	252 ° days AMP
Prefertilisation	ATP	0.007 0.976																			
	ADP	0.255 0.253	0.411 0.058																		
	AMP	0.138 0.54	**0.717** **0**	0.14 0.536																	
	EC	-0.004 0.986	**-0.57** **0.006**	-0.186 0.407	**-0.918** **0**																
Postfertilisation	ATP	-0.106 0.615	0.236 0.29	0.164 0.464	0.249 0.264	-0.181 0.42															
	ADP	**0.608** **0.001**	0.007 0.977	0.235 0.293	-0.109 0.629	0.102 0.65	0.138 0.509														
	AMP	**-0.423** **0.035**	0.129 0.567	-0.269 0.225	0.084 0.71	-0.165 0.463	0.075 0.72	-0.291 0.158													
	EC	0.303 0.141	0.009 0.97	0.209 0.351	0.063 0.782	0.055 0.808	0.366 0.072	0.129 0.538	**-0.817** **0**												
Hatch	ATP	0.202 0.664	-0.41 0.419	-0.522 0.288	0.329 0.525	-0.318 0.539	0.009 0.984	0.195 0.675	-0.419 0.35	0.368 0.417											
	ADP	0.401 0.372	-0.366 0.475	-0.618 0.191	0.64 0.171	-0.693 0.127	-0.357 0.431	0.426 0.34	-0.417 0.352	0.231 0.618	**0.889** **0.007**										
	AMP	0.644 0.118	0.624 0.185	-0.089 0.867	0.767 0.075	-0.731 0.099	-0.254 0.583	**0.812** **0.027**	**-0.785** **0.037**	0.558 0.193	0.424 0.343	0.591 0.162									
	EC	0.012 0.98	-0.06 0.91	0.507 0.305	**-0.898** **0.015**	**0.866** **0.026**	0.554 0.197	-0.269 0.56	-0.548 0.203	0.728 0.063	-0.131 0.78	-0.358 0.43	0.113 0.809								
126 ° days	ATP	-0.025 0.958	0.724 0.104	-0.225 0.668	0.768 0.075	-0.448 0.373	-0.261 0.572	0.469 0.288	0.228 0.622	-0.394 0.382	-0.096 0.856	0.081 0.879	0.351 0.495	-0.715 0.11							
	ADP	0.015 0.975	0.556 0.252	-0.253 0.629	0.79 0.062	-0.571 0.236	-0.48 0.275	0.3 0.514	0.457 0.302	-0.637 0.124	-0.13 0.806	0.097 0.856	0.193 0.714	**-0.886** **0.019**	**0.928** **0.003**						
	AMP	0.26 0.573	-0.243 0.642	0.405 0.426	-0.463 0.355	0.12 0.821	-0.349 0.443	-0.172 0.712	-0.265 0.566	0.205 0.659	-0.512 0.3	-0.285 0.584	0.038 0.943	0.424 0.402	-0.431 0.335	-0.365 0.421					
	EC	0.28 0.543	-0.386 0.45	0.548 0.26	**-0.849** **0.032**	0.478 0.337	0.141 0.764	-0.024 0.959	**-0.812** **0.026**	**0.835** **0.019**	0.059 0.912	0.036 0.947	0.335 0.517	**0.833** **0.039**	-0.645 0.117	**-0.785** **0.037**	0.652 0.113				
252 ° days	ATP	0.418 0.23	-0.042 0.915	-0.153 0.694	0.061 0.876	-0.057 0.884	-0.396 0.257	0.265 0.459	0.57 0.085	**-0.702** **0.024**	-0.327 0.474	-0.095 0.839	-0.367 0.418	-0.518 0.234	0.033 0.943	0.256 0.579	0.092 0.844	-0.456 0.304			
	ADP	0.58 0.079	-0.096 0.806	-0.277 0.471	0.129 0.741	-0.092 0.815	-0.553 0.097	0.448 0.194	0.222 0.538	-0.443 0.2	-0.143 0.76	0.224 0.629	0.109 0.816	-0.451 0.31	0.093 0.842	0.278 0.546	0.269 0.559	-0.24 0.604	**0.888** **0.001**		
	AMP	-0.591 0.072	-0.251 0.514	-0.066 0.867	-0.622 0.074	0.639 0.064	0.533 0.112	**-0.657** **0.039**	**0.726** **0.018**	-0.432 0.213	-0.411 0.36	-0.671 0.099	**-0.941** **0.002**	0.042 0.929	-0.206 0.657	-0.115 0.805	-0.219 0.637	-0.387 0.391	0.153 0.674	-0.249 0.487	
	EC	**-0.634** **0.049**	-0.048 0.901	-0.025 0.95	-0.518 0.153	0.619 0.076	**0.64** **0.046**	**-0.695** **0.026**	0.306 0.39	0.007 0.985	-0.258 0.576	-0.606 0.149	-0.726 0.065	0.272 0.555	-0.218 0.638	-0.276 0.549	-0.221 0.634	-0.074 0.874	-0.33 0.352	**-0.643** **0.045**	**0.823** **0.003**

5.4.2 Haddock

5.4.2.1 First spawning season - 2002

5.4.2.1.1 Adenine nucleotide content

Adenine nucleotide contents were measured in haddock egg and larval samples from the control and nucleotide dietary groups and EC calculated at each stage.

The concentration of ATP, ADP and AMP in eggs did not differ between the control and the nucleotide diets ($F = 0.072$, $p = 0.791$; $F = 0.02$, $p = 0.887$; $F = 0.07$, $p = 0.791$ respectively) (Table 5. 6) when measured on a per gram basis and initially only AMP content was positively correlated to date along the spawning season ($r = 0.34$, $p = 0.049$; Figure 5. 5). However, when adenine nucleotide levels were expressed as nmol per egg as in previous studies (Wendling *et al.* 2000), not only AMP but also ATP levels changed over season ($r = 0.351$, $p = 0.049$; $r = -0.369$, $p = 0.038$ respectively). Energy charge of eggs was not significantly different between both diets (C: 0.75 ± 0.01; NT: 0.75 ± 0.02; $F = 0.00$, $p = 0.953$). However, EC decreased over season significantly ($r = -0.501$, $p = 0.003$; Figure 5. 6).

Table 5. 6 Adenine nucleotide content in haddock egg and larval samples in 2002. Expressed as µmol / g (dry weight). No significant differences were found between dietary treatments. Mean ± SEM.

	Egg		At Hatch		At 5 DPH	
	Control (n=19)	Nucleotide (n=18)	Control (n=15)	Nucleotide (n=14)	Control (n=13)	Nucleotide (n=14)
ATP[1]	0.807 ± 0.044	0.822 ± 0.03	1.602 ± 0.22	1.848 ± 0.348	2.907 ± 0.33	2.330 ± 0.383
ADP[1]	0.351 ± 0.03	0.358 ± 0.03	0.975 ± 0.14	1.271 ± 0.208	0.914 ± 0.118	0.791 ± 0.101
AMP[1]	0.162 ± 0.019	0.172 ± 0.029	0.340 ± 0.062	0.377 ± 0.079	0.272 ± 0.039	0.247 ± 0.054
EC	0.75 ± 0.01	0.75 ± 0.02	0.71 ± 0.03	0.70 ± 0.03	0.8 ± 0.03	0.79 ± 0.02

[1]: µmol / g (dry weight)

No significant differences between diets were found in the ATP, ADP and AMP content of larval samples at hatch and at 5 DPH (Table 5. 6). ATP, ADP and AMP were not significantly correlated to date at any larval stage (Table 5. 7). Energy charge of larval samples was not significantly different between diets at any of the two larval stages sampled (hatch: $F = 0.08$, $p = 0.773$; 5 DPH: $F = 0.05$, $p = 0.832$; Table 5. 6). Therefore, EC from both dietary treatments were pooled and the variation studied along the season. Energy charge at hatch and at 5 DPH were not correlated with date, indicating no seasonal trend ($p = 0.182$; $p = 0.807$ respectively).

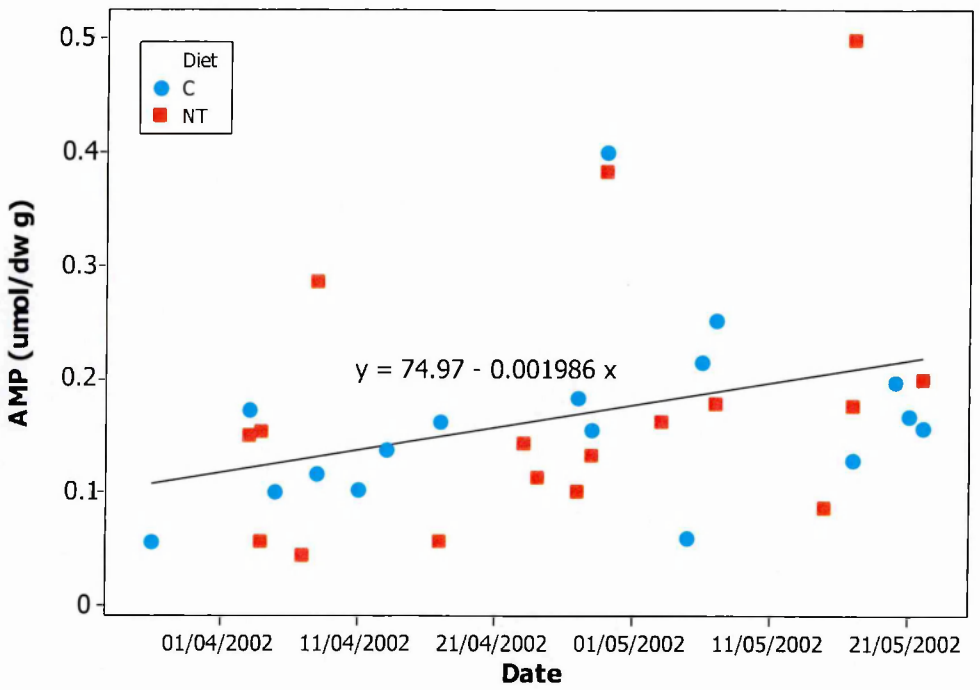


Figure 5. 5 Variation of the AMP content in haddock eggs in the 2002 spawning season. Control (C): Blue circles, Nucleotide (NT): red squares.

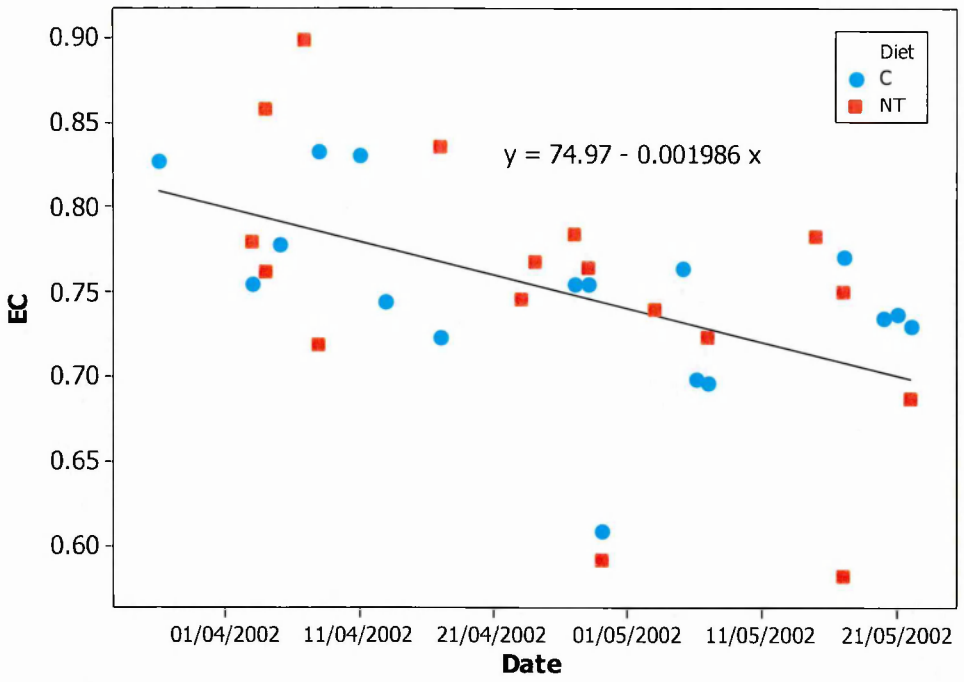


Figure 5. 6 Variation of the energy charge (EC) in haddock eggs in the 2002 spawning season. Control (C): Blue circles, Nucleotide (NT): red squares.

Control and nucleotide data were then pooled and variation of ATP, ADP, and AMP between egg and larval stages was studied. ATP content increased from the egg to the end of yolk absorption ($F = 29.44$, $p < 0.001$), while ADP and AMP increased from egg to hatch ($T = 7.837$, $p < 0.001$; $T = 4.300$, $p < 0.001$) but did not change at 5 DPH ($T = 1.078$, $p = 0.208$; $T = 2.024$, $p = 0.113$) (Figure 5. 7). Energy charge values from both diets were also pooled and appeared to remain stable until hatch ($p = 0.143$), however increased significantly thereafter until 5 DPH ($p = 0.002$).

Relations with other egg and larval parameters

Egg EC was positively correlated with larval survival at 5 DPH ($r = 0.587$, $p = 0.021$; Figure 5. 8). Egg EC was also positively correlated with larval dry weight at hatch ($r = 0.578$, $p = 0.031$) and larval length at 5 DPH ($r = 0.583$, $p = 0.047$).

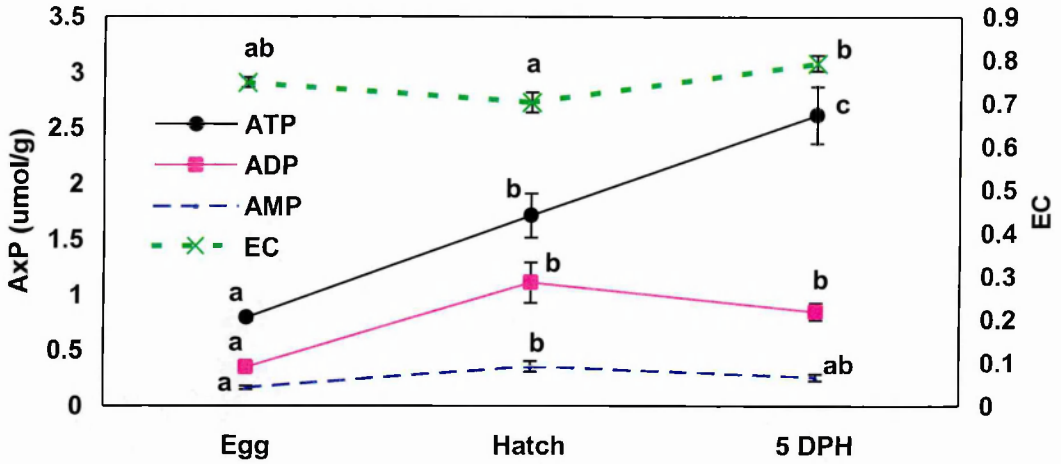


Figure 5. 7 Variation of adenine nucleotides and energy charge (EC) in haddock eggs and larvae until the end of yolk resorption in the 2002 spawning season. Data points with different letters of the same colour indicate a significant difference. Mean ± SEM

Table 5. 7 Correlations of adenine nucleotides (ATP, ADP & AMP) and energy charge (EC) in haddock egg and larval samples along the 2002 spawning season. Significant correlations are denoted in bold format.

Cell content:	r - value p - value		Egg				Hatch				5 DPH		
		DATE	ATP	ADP	AMP	EC	ATP	ADP	AMP	EC	ATP	ADP	AMP
Egg	ATP	-0.269 0.123											
	ADP	0.264 0.132	**0.494** **0.003**										
	AMP	**0.34** **0.049**	0.157 0.377	**0.697** **0**									
	EC	**-0.501** **0.003**	0.082 0.643	**-0.722** **0**	**-0.913** **0**								
Hatch	ATP	-0.105 0.596	-0.006 0.985	0.4 0.197	0.152 0.636	-0.27 0.397							
	ADP	0.011 0.957	-0.119 0.714	0.348 0.268	0.181 0.573	-0.255 0.424	0.185 0.347						
	AMP	0.291 0.133	-0.163 0.614	0.324 0.304	0.081 0.804	-0.196 0.542	0.172 0.382	**0.57** **0.002**					
	EC	-0.26 0.182	0.217 0.499	0.052 0.873	0.021 0.947	-0.01 0.975	**0.675** **0**	**-0.424** **0.024**	**-0.55** **0.002**				
5 DPH	ATP	-0.081 0.695	-0.095 0.795	-0.039 0.915	0.244 0.497	-0.234 0.516	**-0.47** **0.024**	-0.207 0.344	-0.35 0.102	-0.214 0.326			
	ADP	-0.122 0.551	**0.752** **0.012**	0.348 0.324	-0.463 0.178	0.596 0.069	-0.07 0.751	-0.101 0.648	-0.054 0.808	-0.035 0.874	0.337 0.092		
	AMP	-0.286 0.156	0.575 0.082	0.447 0.195	-0.385 0.272	0.423 0.223	0.145 0.509	0.109 0.619	0.226 0.299	-0.01 0.964	-0.075 0.716	0.353 0.077	
	EC	-0.05 0.807	-0.418 0.229	-0.228 0.526	0.505 0.137	-0.524 0.12	-0.336 0.118	-0.225 0.302	**-0.441** **0.035**	-0.019 0.93	**0.775** **0**	0.014 0.945	**-0.554** **0.003**

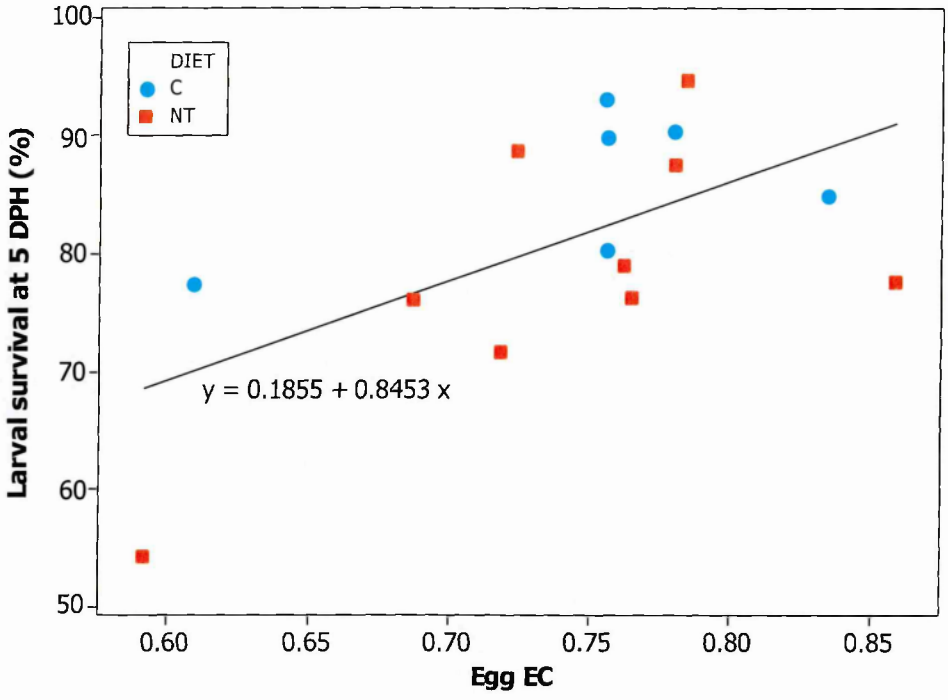


Figure 5. 8 Scatterplot of larval survival at 5 days post hatch (DPH) against energy charge (EC) of haddock eggs in the 2002 spawning season. Control (C): Blue circles, Nucleotide (NT): red squares.

5.4.2.1.2 *Total potentially available nucleotide content in haddock*

Adenosine, guanosine, cytidine, uridine and inosine content in egg samples was similar in both diets and no significant differences were found (Ado: F = 0.35, p = 0.559; Guo: F = 0.09, p = 0.761; Cyd: F = 0.24, p = 0.625; Urd: F = 0.40, p = 0.533 and Ino: F = 0.01, p = 0.909; Table 5. 8). Total nucleoside content in eggs from the nucleotide diet averaged 77.86 ± 6.73 µmol / sample g, compared to 81.75 ± 6.92 µmol / sample g in control eggs. However, the difference was not significant (F = 0.27, p = 0.605). The concentration of all the different nucleosides (except inosine) in the eggs decreased over the spawning season (Table 5. 9) and therefore total nucleoside content also decreased (r = - 0.641, p < 0.001; Figure 5. 9).

Table 5. 8 Nucleoside (NS) content in haddock egg and larval samples in 2002. Expressed as µmol / sample g (dry weight). No significant differences were found between dietary treatments. Mean ± SEM.

	Egg		At Hatch		At 5 DPH	
	Control (n=18)	Nucleotide (n=17)	Control (n=14)	Nucleotide (n=15)	Control (n=13)	Nucleotide (n=14)
Adenosine[1]	19.94 ± 2.12	18.13 ± 2.19	78.87 ± 12.15	52.60 ± 6.43	54.69 ± 6.24	42.08 ± 3.18
Guanosine[1]	17.86 ± 1.77	17.12 ± 1.77	120.22 ± 15.68	96.07 ± 9.74	161.10 ± 18.89	132.61 ± 12.29
Cytidine[1]	8.31 ± 0.70	7.85 ± 0.66	n.d.	n.d.	48.76 ± 7.40	41.28 ± 3.66
Uridine[1]	11.63 ± 0.74	10.97 ± 0.70	62.29 ± 6.22	47.54 ± 4.67	62.29 ± 6.22	47.54 ± 4.67
Inosine[1]	24.01 ± 1.60	23.79 ± 1.42	132.09 ± 12.08	137.68 ± 9.54	178.69 ± 19.13	145.29 ± 14.28
Total [1]	81.75 ± 6.92	77.86 ± 6.73	393.46 ± 46.13	333.89 ± 30.39	505.52 ± 57.88	408.80 ± 38.07

[1]: µmol / sample g (dry weight);
n.d. : non detectable

In larval samples, cytidine levels were very low and not detected by the HPLC at hatch. No significant differences between diets in the content of any of the other nucleosides were found at hatch (Ado: F = 2.75, p = 0.110; Guo: F = 1.80, p = 0.192; Urd: F = 0.10, p = 0.756; Ino: F =0.13, p = 0.718; Table 5. 8). Levels of adenosine and guanosine in yolk-sac larvae decreased along the spawning season (r = - 0.73, p < 0.001; r = - 0.544, p = 0.003, respectively) whereas inosine and uridine were not correlated with date (p = 0.58; p = 0.338, respectively). Nevertheless, as in the egg samples, the total nucleoside content in yolk-sac larval samples significantly decreased along the season (Table 5. 9).

Table 5. 9 Correlations of nucleosides (Adenosine, guanosine, cytidine, inosine, uridine) in haddock egg and larval samples versus date along the 2002 spawning season. Significant correlations are denoted in bold format.

Cell contents: r - value			Egg						Hatch					5 DPH				
p - value		DATE	Adenosine	Guanosine	Cytidine	Inosine	Uridine	NS-Total	Adenosine	Guanosine	Inosine	Uridine	NS-Total	Adenosine	Guanosine	Cytidine	Inosine	Uridine
Egg	Adenosine	**-0.755**																
		0																
	Guanosine	**-0.74**	**0.985**															
		0	**0**															
	Cytidine	**-0.581**	**0.881**	**0.922**														
		0	**0**	**0**														
	Inosine	-0.214	**0.594**	**0.678**	**0.799**													
		0.217	**0**	**0**	**0**													
	Uridine	**-0.481**	**0.846**	**0.893**	**0.943**	**0.912**												
		0.003	**0**	**0**	**0**	**0**												
	NS-Total	**-0.641**	**0.949**	**0.977**	**0.958**	**0.809**	**0.965**											
		0	**0**	**0**	**0**	**0**	**0**											
Hatch	Adenosine	**-0.73**	**0.611**	**0.661**	**0.835**	**0.666**	**0.78**	**0.735**										
		0	**0.027**	**0.014**	**0**	**0.013**	**0.002**	**0.004**										
	Guanosine	**-0.544**	0.426	0.438	0.55	0.348	0.5	0.47	**0.916**									
		0.003	0.147	0.135	0.051	0.244	0.082	0.105	**0**									
	Inosine	-0.112	-0.007	-0.002	0.05	-0.008	0.049	0.005	**0.564**	**0.749**								
		0.58	0.982	0.996	0.871	0.979	0.874	0.988	**0.002**	**0**								
	Uridine	-0.192	0.135	0.225	0.427	0.368	0.398	0.285	**0.63**	**0.743**	**0.891**							
		0.338	0.66	0.461	0.146	0.216	0.179	0.346	**0**	**0**	**0**							
	NS-Total	**-0.486**	0.383	0.414	**0.553**	0.39	0.511	0.459	**0.896**	**0.973**	**0.859**	**0.851**						
		0.01	0.197	0.16	**0.05**	0.188	0.074	0.115	**0**	**0**	**0**	**0**						
5 DPH	Adenosine	0.054	-0.326	-0.246	0.226	0.44	0.204	-0.038	0.354	**0.4**	0.211	0.268	0.353					
		0.798	0.328	0.467	0.504	0.176	0.547	0.912	0.082	**0.047**	0.311	0.195	0.083					
	Guanosine	0.017	-0.105	0.05	0.479	**0.681**	0.444	0.24	**0.397**	**0.416**	0.192	0.27	0.365	**0.92**				
		0.937	0.758	0.883	0.136	**0.021**	0.171	0.476	**0.049**	**0.039**	0.358	0.192	0.073	**0**				
	Cytidine	0.179	-0.34	-0.24	0.202	0.294	0.078	-0.099	0.069	-0.03	-0.246	0.012	-0.089	**0.923**	**0.934**			
		0.541	0.41	0.566	0.632	0.479	0.855	0.816	0.813	0.919	0.396	0.968	0.761	**0**	**0**			
	Inosine	0	-0.104	0.008	0.406	**0.617**	0.391	0.199	0.38	0.388	0.184	0.194	0.34	**0.888**	**0.962**	**0.913**		
		0.998	0.76	0.98	0.215	**0.043**	0.235	0.557	0.061	0.055	0.379	0.354	0.096	**0**	**0**	**0**		
	Uridine	-0.149	-0.161	-0.035	0.404	0.6	0.373	0.159	**0.443**	**0.443**	0.217	0.232	0.394	**0.735**	**0.677**	**0.967**	**0.645**	
		0.477	0.636	0.918	0.217	0.051	0.259	0.64	**0.027**	**0.026**	0.298	0.264	0.051	**0**	**0**	**0**	**0.001**	
	NS-Total	0.012	-0.137	-0.003	0.418	**0.622**	0.389	0.186	0.391	**0.417**	0.205	0.269	0.368	**0.946**	**0.984**	**0.952**	**0.951**	**0.758**
		0.956	0.688	0.993	0.2	**0.041**	0.237	0.583	0.053	**0.038**	0.327	0.193	0.07	**0**	**0**	**0**	**0**	**0**

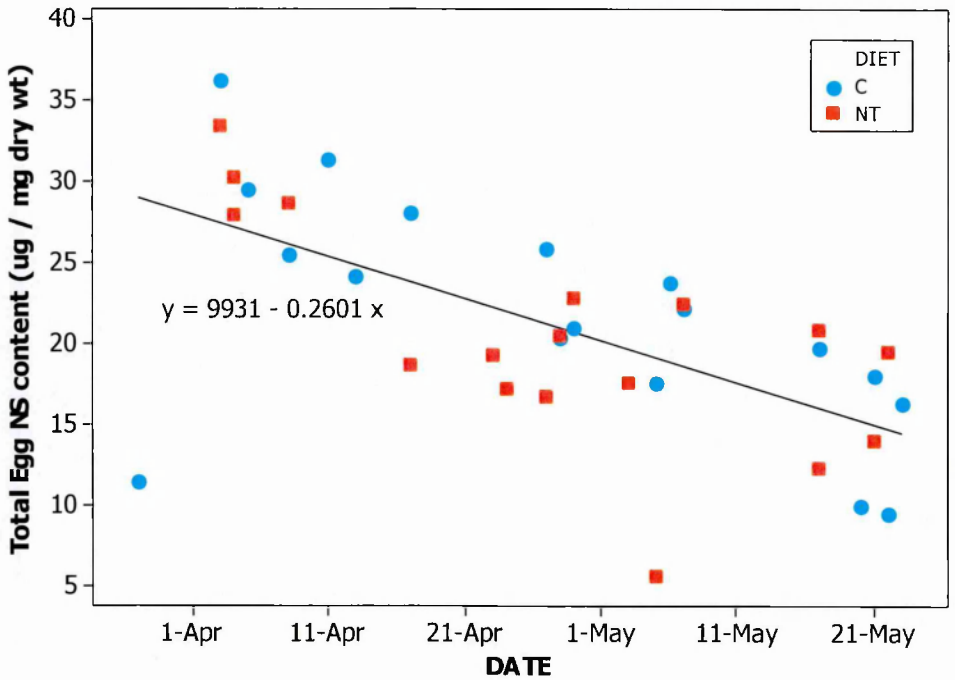


Figure 5. 9 Variation of the total nucleoside (NS) content in haddock eggs against date in the 2002 spawning season. Control (C): Blue circles, Nucleotide (NT): red squares.

Five days after hatch, the larval nucleoside content did not differ between dietary treatments. Mean values of each nucleoside were slightly lower in samples from the nucleotide group, however differences were not significant (Ado: $F = 2.37$, $p = 0.137$; Guo: $F = 1.65$, $p = 0.212$; Cyd: $F = 1.05$, $p = 0.325$; Urd: $F = 3.67$, $p = 0.068$; Ino: $F = 2.00$, $p = 0.171$; Table 5. 8). Total nucleoside content was not significantly different between the experimental diets ($F = 1.95$, $p = 0.176$; Table 5. 8). No seasonal variation pattern was detected along the spawning season for the different nucleotides; all correlations with 'date' were not significant (Table 5. 9).

5.4.2.2 Second spawning season - 2003

Adenine nucleotide content was measured again in haddock egg and larval samples from the control and nucleotide dietary groups during the second spawning season and the EC calculated at each stage.

In the egg samples, ATP, ADP and AMP contents were not significantly different between the control and the nucleotide-enhanced diets (ATP: $F = 0.26$, $p = 0.615$; ADP: $F = 0.02$, $p = 0.881$; AMP: $F = 0.01$, $p = 0.909$; Table 5. 10). General linear model analysis further demonstrated that there was no diet effect, however ATP concentration in eggs decreased over the spawning season ($F = 9.51$, $p = 0.006$; Figure 5. 10, Table 5. 11).

Table 5. 10 Adenine nucleotide content and energy charge (EC) in haddock egg and larval samples in 2003. Expressed as µmol / g (dry weight). No significant differences were found between dietary treatments. Mean ± SEM.

	Egg		Larvae at hatch	
	Control (n = 15)	Nucleotide (n = 12)	Control (n = 3)	Nucleotide (n = 3)
ATP[1]	0.819 ± 0.053	0.775 ± 0.072	0.789 ± 0.33	0.318 ± 0.097
ADP[1]	0.275 ± 0.021	0.268 ± 0.042	0.771 ± 0.070	0.521 ± 0.095
AMP[1]	0.078 ± 0.007	0.099 ± 0.022	0.276 ± 0.060	0.120 ± 0.028
EC	0.81 ± 0.01	0.80 ± 0.02	0.62 ± 0.09	0.60 ± 0.01

[1]: µmol / g (dry weight)

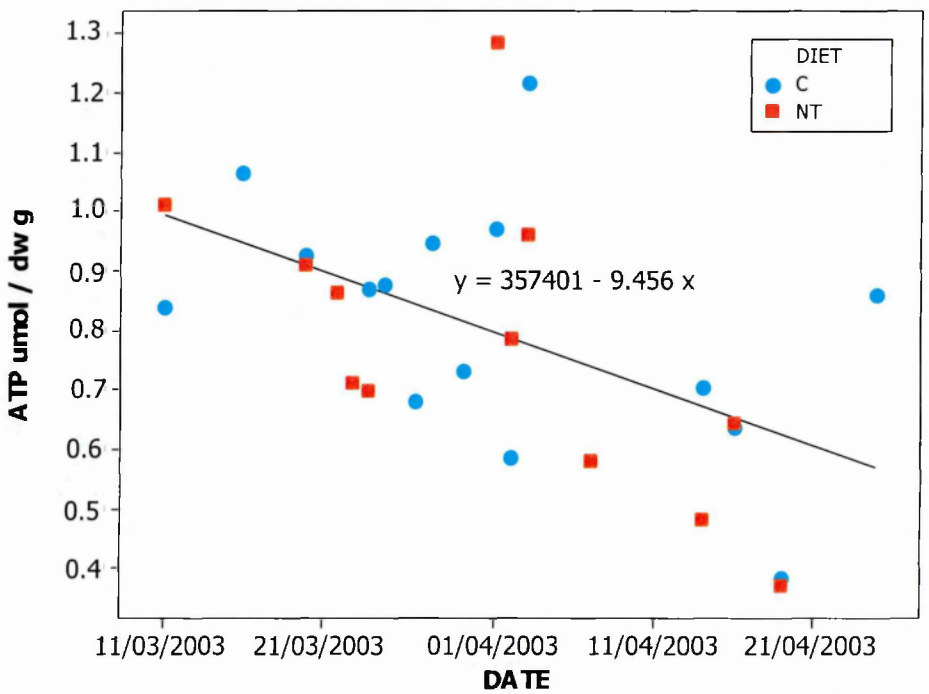


Figure 5. 10 Variation in ATP content of haddock eggs during the 2003 spawning season. Control (C): Blue circles, Nucleotide (NT): red squares.

Table 5. 11 GLM analysis of the ATP content of haddock eggs in the 2003 spawning season. Significant differences are denoted by *. R^2 = 44.53 %, R^2adj = 31.32 %.

Source	DF	Sums of Squares	Mean Squares	F Value	P Value
Diet	1	2009	2009	0.06	0.812
Tank (Diet)	3	218343	72781	2.11	0.129
Date	1	327534	327534	9.51	0.006*
Error	21	723514	34453		

Energy charge in eggs from the control group averaged 0.81 ± 0.01, which was not significantly different to the mean value in the nucleotide group, 0.80 ± 0.02 (F = 0.24, p = 0.629). As in the previous spawning season, regression linear analysis showed that egg EC also decreased over the season (r = - 0.474, p = 0.013; Figure 5. 11).

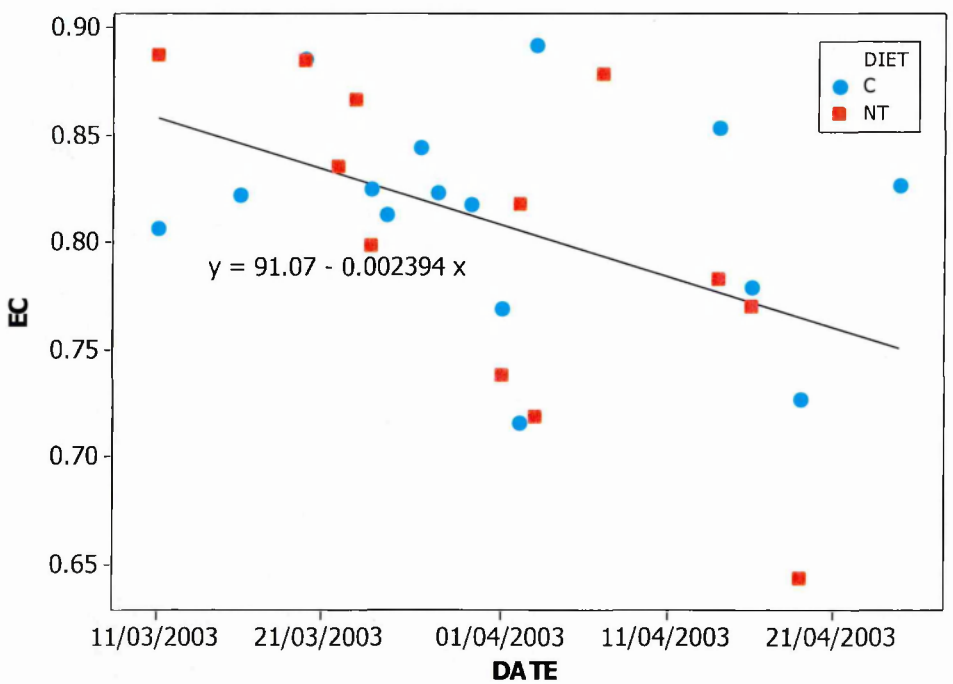


Figure 5. 11 Variation of energy charge (EC) in haddock eggs during the 2003 spawning season. Control (C): Blue circles, Nucleotide (NT): red squares.

In the second spawning season, adenine nucleotide content in larval samples was analysed only at hatch. However, ATP, ADP and AMP content were not significantly different between dietary treatments (F = 1.19, p = 0.355; F = 4.75, p = 0.118; F = 3.75, p = 0.148 respectively;

Table 5. 10). No significant correlations were found over the season in the larval samples (Table 5. 12)

Control and nucleotide data were then pooled and variation of ATP, ADP, AMP and EC between egg and larval samples at hatch was studied (Figure 5. 12). Changes in ATP content were not significant ($F = 2.23$, $p = 0.164$), ADP increased from egg to hatch ($F = 29.88$, $p < 0.001$) as AMP also did ($F = 11.80$, $p = 0.02$). Energy charge decreased from 0.81 ± 0.01 in fertilised eggs to 0.61 ± 0.05 in larvae after hatch ($F = 24.66$, $p < 0.001$).

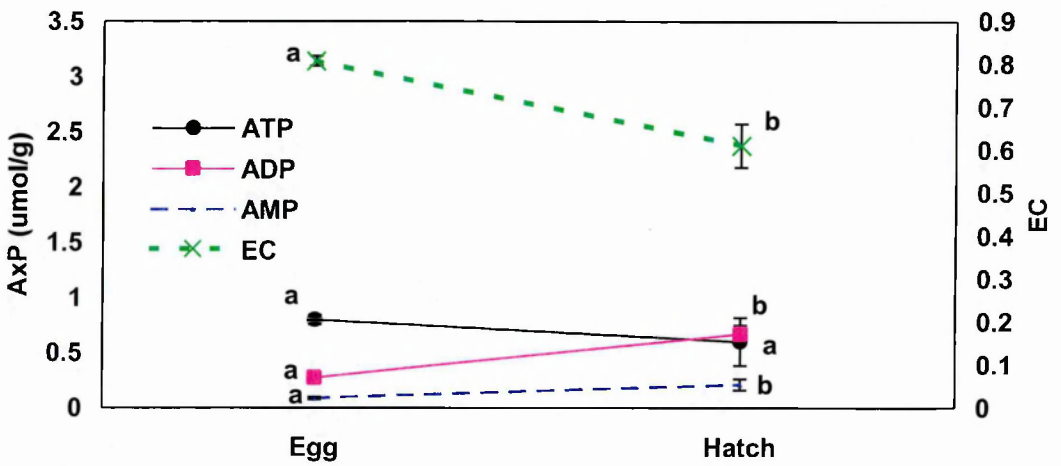


Figure 5. 12 Variation of adenine nucleotides and energy charge (EC) in haddock eggs and larvae at hatch in the 2003 spawning season. Y-axis scale as in Figure 5. 7. Data points with different letters of the same colour indicate a significant difference. Mean ± SEM

Relations with other egg and larval parameters

ATP level of haddock eggs were not correlated ($p = 0.590$) to the fraction of floating eggs (viable eggs). However, the energy charge of haddock eggs was almost positively correlated to the fraction of floating eggs ($r = 0.369$, $p = 0.058$), while ADP and AMP levels were negatively correlated with the viable fraction ($r = - 0.432$, $p = 0.024$; $r = - 0.521$, $p = 0.005$).

Table 5. 12 Correlations of adenine nucleotides (ATP, ADP & AMP), energy charge (EC) in egg and larval samples along the 2003 spawning season. Significant correlations are denoted in bold format.

Cell content: r - value			Egg				Hatch		
	p - value	Date	ATP	ADP	AMP	EC	ATP	ADP	AMP
Egg	ATP	**-0.516** **0.006**							
	ADP	-0.02 0.919	**0.471** **0.013**						
	AMP	0.148 0.461	0.252 0.204	**0.777** **0**					
	EC	**-0.474** **0.013**	**0.388** **0.045**	**-0.52** **0.005**	**-0.725** **0**				
Hatch	ATP	0.441 0.457	-0.639 0.246	**0.946** **0.015**	0.794 0.108	**-0.96** **0.01**			
	ADP	0.506 0.384	0.352 0.562	0.236 0.702	-0.192 0.756	0.083 0.895	0.131 0.833		
	AMP	0.676 0.21	0.658 0.227	0.127 0.839	-0.151 0.809	0.234 0.705	-0.108 0.863	**0.88** **0.049**	
	EC	0.002 0.997	-0.858 0.063	0.704 0.185	0.68 0.206	**-0.886** **0.046**	**0.879** **0.05**	-0.271 0.659	-0.561 0.325

5.5 Discussion

This part of the project was carried out in order to study the potential influence of nucleotide-enhanced broodstock diets on the nucleotide content and adenylate energy charge of halibut and haddock eggs and larvae. Energy charge of fertilised halibut eggs was significantly higher in the nucleotide treatment, however no differences between dietary treatments were found in haddock eggs. Energy charge of eggs decreased during both haddock spawning seasons and is suggested to be a reliable indicator of survivorship during embryo development as in carp (Boulekbache *et al.*, 1989). The provision of an extra supply of nucleotides to the broodstock through their diets did not cause any increment in the content of these in halibut and haddock eggs and larvae as expected. The fact that the nucleotide-enhanced diets were administrated to broodfish with no concominant change in the total nucleotide content of eggs and larvae may suggest that adult fish utilised this nucleotide 'boost' to cover their own nutritional requirements during oogenesis and reproduction.

Variation in ATP, ADP, AMP and EC content during larval development are discussed in relation to the different substrates used for catabolic oxidation by halibut and haddock larvae from hatch.

5.5.1 Egg samples

As stated by Vetter *et al.* (1983), comparisons with adenine nucleotide concentration and EC values of eggs reported in other fish studies should be made with caution, since many results are conflicting, sample preparation method may be inaccurate or even not described (Boulekbache, 1981). Furthermore, in some cases not all the concentrations of the different adenine nucleotides are reported (Wendling *et al.*, 2000; 2004).

The ATP content of freshly collected and unfertilised halibut eggs was similar in both dietary treatments. Wendling *et al.* (2000) reported ATP levels of 2.61 ± 0.14 nmol of ATP per egg in chinook salmon (*Oncorhynchus tshawytscha*), which is approximately three times greater than that in the present study (0.864 ± 0.052 nmol ATP per egg); this difference probably being due to egg size (diameter: 6.0 – 7.0 mm vs 3.2 mm in salmon and halibut respectively). In the present study, ATP levels in halibut eggs from the control group tended to decrease (from 0.57 ± 0.05 to 0.47 ± 0.04) after fertilisation, whereas they remained constant in the nucleotide group (from 0.55 ± 0.02 to 0.54 ± 0.02). ADP and AMP levels also appeared to decrease slightly after fertilisation in eggs from both groups. As a result of these alterations in the levels of adenine nucleotides, the EC decreased after fertilisation in the control group while remaining constant and significantly higher in the nucleotide eggs. In haddock however, the effect of fertilisation could not be studied since eggs were collected once fertilised and most eggs were already in the first cleavage stages. Depletion of ATP levels immediately after fertilisation have been reported previously in Chinook salmon (Wendling *et al.,* 2000) and rainbow trout (Wendling *et al.*, 2004). Both studies found that ATP levels decreased in unfertilised eggs after water activation. Since the reduction in ATP content following activation, with or without fertilisation, were similar in the timing and extent of decline, it was concluded that fertilisation, per se, did not cause the immediate decrease of ATP. This phenomenon was explained instead as a result of water activation of the egg, suggesting that the events associated with egg activation (such as intracellular/cytoskeletal changes) are energy-consuming. Since ATP is used at the moment of activation/fertilisation, the ATP content of unfertilised eggs might correlate with quality/fertility of released eggs. In the present study, ATP levels in unfertilised halibut eggs were not correlated to fertilisation success (measured as fertilisation rate), nor were they for rainbow trout eggs (Wendling *et al.* 2004). However, drop-out 24h after fertilisation was almost correlated to ATP levels prior to and after fertilisation ($r = -0.414$, $p = 0.099$; $r = -0.434$, $p = 0.056$). Although not significant, it might be possible to infer that egg survival 24h after fertilisation may be affected by their ATP levels, since the highest egg mortalities occurred in those egg batches with the lowest ATP concentration. Furthermore, when ATP content was expressed as nmol per egg, ATP levels were negatively correlated to drop-out after fertilisation ($r = -0.468$, p

= 0.043), supporting this hypothesis. Moreover, the batches which suffered the lowest egg mortalities after fertilisation had the highest ATP levels in larval samples at hatch (r = - 0.846, p = 0.017).

Lahnsteiner & Patarnello (2003) found no significant difference in the ATP levels between viable and non-viable seabream eggs, although EC was significantly higher in viable eggs. Unfortunately, no correlations were found between the EC levels of halibut and haddock eggs with drop-out rates in the present study. Energy charge of haddock eggs did not differ between diets in 2002 (EC = 0.75 approx.) or 2003 (EC = 0.80 approx.). In most fish so far studied, the energy metabolism of eggs is characterised by a relatively high energy charge value (Table 5. 13). Milman & Yurowitzky (1973) and Lahnsteiner & Patarnello (2003) reported that EC values remained constant throughout the embryonic development of loach and seabream eggs respectively, whereas reductions were described from 0.72 to 0.61 from fertilisation to closure of blastopore in trout (Boulekbache, 1981) and from 0.87 to 0.58 from fertilisation to just before hatching in red drum (Vetter *et al.*, 1983). This supports the hypothesis suggested by Boulekbache (1981) of two types of strategies during embryonic development in teleosts: stable versus varying EC levels. Variation in EC during embryonic development was not studied in the present project therefore it is not possible to know what sort of strategy halibut and haddock follow. Nevertheless, the energy charge of halibut and haddock eggs was of the same range as those reported previously (Table 5. 13), suggesting that for teleost eggs, in general EC's are lower than those in other oviparous vertebrates (*Bufo arenarum*: EC = 0.93 and *Xenopus laevis*: EC = 0.94; Moreno *et al.*, 1976 and Thoman & Gerhart, 1979 respectively), often being below 0.80, a level indicating stress in many organisms (Boulekbache, 1981; Vetter *et al.*, 1983).

Table 5. 13 Energy charge (EC) ratios of fertilised eggs reported in previous studies.

Common name	Species	EC	Reference
Loach	*Misgurnus fossilis*	0.73	Milman & Yurowitsky, 1973
Trout	Not specified	0.72	Boulekbache, 1981
Red drum	*Sciaenops ocellata*	0.87	Vetter *et al.*, 1983
Carp (in oocytes)	*Cyprinus carpio*	0.85	Boulekbache *et al.*, 1989
Gilthead seabream	*Sparus aurata*	0.75	Lahnsteiner & Patarnello, 2003
Atlantic halibut	*Hippoglossus hippoglossus*	0.78	Present study
Haddock	*Melanogrammus aeglefinus*	0.75 – 0.81	Present study

Energy charge in haddock eggs decreased during the 2002 and 2003 spawning seasons. This is the first study to report on the variation of EC in fish eggs during a spawning season. Similar alterations had been described in seabass spermatozoa (Dreanno *et al.*, 1999). Energy charge decreased from the beginning towards the end of the season and was associated with a depletion of ATP and increase of AMP in spermatozoa by the end of the season. In this study, seasonal changes of the EC ratio were also a consequence of decreased ATP and increased AMP levels in the eggs at the end of the 2002 spawning season, although this can only be linked to decreased ATP at the end of the following season. Dreanno *et al.* (1999) found that EC and ATP reductions were linked to alterations of the motility and fertilisation capacity of the spermatozoa; all occurring as a result of the ageing process of the seabass milt throughout the season. Haddock are easily subject to stress if handled during spawning so as mentioned previously, in the present study eggs were collected when already fertilised. Therefore, changes in the EC and adenine nucleotides in the haddock milt or unfertilised eggs as a result of ageing or time in the season could not be reported. Instead, EC was measured in fertilised eggs (morula stage) and almost correlated ($p = 0.058$) to the amount of floating eggs after collection (approximately 12-24h from spawning) in 2003. It may be inferred (with a 94.2% confidence) from these results that egg batches with the highest EC had the largest fraction of floating/viable eggs. The fact that the floating egg fraction decreased during the 2003 spawning season and that ADP and AMP levels were higher in batches with the smallest floating fraction supports this hypothesis. Furthermore, Boulekbache *et al.* (1989) reported decreasing ATP and EC during the ageing process of carp oocytes which were associated with reduced content of ATP and EC in carp embryos and those with the lowest EC suffered the highest mortalities. Energy charge in eggs from 2002 was not correlated with egg viability after collection in the first spawning season of haddock. As reported in chapter 2, the floating egg fraction followed a dome-shape distribution, whereas EC decreased along the season. However, egg EC was correlated to larval survival at the end of the yolk absorption period. Energy charge level measured in the early embryonic stages (morula) is then suggested as a suitable indicator of egg viability during its

development, while further work is suggested to confirm the link found between egg EC and larval survival.

5.5.2 Larval samples

This is a novel study since it is the first of its kind reporting EC values in larvae, particularly through the yolk-sac stage, while previous investigations only focussed on the energy charge of egg samples.

Adenine nucleotide levels (ATP, ADP, AMP) of halibut nucleotide larvae sampled at hatch, 126 ° days or 252 ° days did not differ from those of the control broodstock. ATP levels decreased dramatically from hatch to 126 ° days and then increased slightly at the end of the yolk-sac stage (252 ° days); ADP and AMP also followed a similar trend. This fall in ATP content during the first half of the yolk-sac stage may indicate an inability of ATP production to keep pace with ATP demand in the developing larvae. Developing yolk-sac halibut larvae use endogenous free amino acids (FAA) as the only energy source during the first two weeks after hatch (0-70 ° days), while catabolic oxidation of proteins together with polar lipids (mainly PC: phosphatidyl choline) and FAA occurs during the following 3 weeks, with neutral lipids (mainly TAG: triacyl-glycerol) also being used shortly before the end of the third week. During the last phase (200-270 ° days) proteins, FAA, and neutral and polar lipids are used (Finn *et al.*, 1995a). The use of FAA and proteins (compounds with a lower energetic content than lipids) as the first fuels by the larvae may account for the initial depletion of ATP, while the increased utilisation of lipids (which have an energy content higher than proteins) as substrates from the middle of the yolk-sac stage may account for the recovery in ATP levels. As a result of the changes in the adenine nucleotide levels, the EC ratio decreased from 0.83 ± 0.02 at hatch to 0.55 ± 0.07 and 0.53 ± 0.06 (for control and nucleotide pooled) at the middle and end of the yolk-sac stage respectively. This low EC at 252 ° days may indicate that halibut larvae were stressed by this stage. Experiments carried out by Lein & Holmefjord (1992) recommended that halibut larvae

should be offered food between 200 and 265 ° days. This was also confirmed in studies on energy metabolism of yolk-sac larvae of halibut by Finn *et al.* (1995a), who showed that yolk absorption was accelerated during the first half of the yolk-sac stage and suggested that exogenous feeding should be applied by 200 ° days post hatch. In our experiment, since halibut larvae were not offered any food during yolk absorption, and yolk was fully absorbed by 252 ° days, larvae may have been subject to nutritional stress conditions which may account for the low EC values described at this stage.

In haddock, ATP, ADP, and AMP content increased from the egg to larval stage at hatch in the 2002 spawning season (Figure 5. 7). However, while ATP levels in larvae increased at the end of yolk absorption (5 DPH), ADP and AMP decreased at this stage. Differences between diets were not statistically significant ($p > 0.05$). Unlike halibut, the increase in ATP content throughout yolk absorption may indicate that ATP production kept pace with ATP demand in the developing larvae and would stimulate rates of catabolism of the yolk reserves at a higher rate. Up to date, no studies have investigated the sequential use of endogenous substrates for catabolic oxidation of developing yolk-sac haddock larvae. However, this has been reported for cod (Finn *et al.*, 1995b), another gadoid fish, with a larval development and yolk-sac stage duration very similar to that of haddock. Finn *et al.* (1995b) reported that unlike halibut, cod larvae already utilise endogenous FAA, PC and TAG at hatch, with proteins used two days after hatch (approx 12 ° days). Supposing that haddock yolk-sac larval metabolism is similar to that of cod, it could be inferred that the utilisation of FAA and neutral and polar lipids before hatching contributed to the increasing levels of ATP during yolk absorption in haddock larvae. As a consequence of the changes in adenine nucleotide levels, energy charge increased to 0.8 at 5 days after hatch (~ 40 ° days), indicating that haddock larvae were in a good condition and not as nutritionally stressed as halibut larvae. Haddock larvae would have been offered live food at 42 – 54 °days if the experiment had continued as suggested by Finn *et al.* (1995b) for cod.

For the 2003 season, as in 2002, the broodstock diet enhanced with nucleotides did not change adenine nucleotide levels or the EC of haddock larvae sampled at hatch (Figure 5. 12). Pooled

contents of ADP and AMP increased when compared to those in egg samples, while ATP appeared to continue stable. The cause behind ATP levels not increasing between the egg and larval stage remains unknown. However, stable levels of ATP from the egg to larval stage with increasing levels of ADP and AMP would explain why the EC significantly decreased at hatch more drastically while statistically remained stable in the previous spawning season (2002). Previous investigations have reported reductions in the egg EC between fertilisation/morula and prior to hatch (e.g. Vetter *et al.*, 1983). However in the present study EC was estimated from just hatched larvae.

Chapter 6: General Discussion & Conclusions

Although the different results were discussed in each chapter, the main goal of this section is to link the observations made in order to form an overall picture of the various effects of the dietary nucleotides on fish reproduction, particularly on spawning performance, egg and larval quality. This is the first study in which nucleotide-enriched diets have been given to broodstock and it has shown that the administration of an external supply of nucleotides in the broodstock diet had beneficial effects on fish reproduction. Similar results were found in Atlantic halibut and haddock, two fish species with different life histories.

6.1 Feeding and fish condition

Previous fish studies have reported feeding to be both affected (e.g. Ishida & Hidaka, 1987) and unaffected (e.g. Peres & Oliva-Teles, 2003), by dietary nucleotides. In this study the halibut and haddock broodstock followed a typical feed intake, with loss of appetite and consequently feeding levels decreasing as the spawning season approached. However, the nucleotide-enriched diet did not affect the feed consumption (neither positively nor negatively) in either species. As suggested earlier, the disparity between this result and others in previous studies may be explained by the different nucleotide composition and their source. Yeast-derived nucleotides at inclusion levels of 0.5 % and 0.8 % were used for halibut and haddock respectively in our study. The condition of the broodstock at the end of the spawning season was similar for both diets in both species.

6.2 Spawning performance

Oogenesis is a process with a high nucleotide requirement as a result of new cells being rapidly formed. Since tissues with a high cell turnover are known to depend on dietary nucleotides for the synthesis of nucleic acids (Navarro *et al.*, 1996), and elevated levels of nucleic acids have been described after exogenous nucleotide supplementation (e.g. Yamauchi *et al.*, 1998), the administration of an external supply of nucleotides through the broodstock diet prior to oogenesis may have increased the availability of these compounds, monomeric units of DNA/RNA, during egg formation. It was hypothesised that diet composition, in terms of nucleotide content, would also be reflected in egg nucleotide levels. However, results from the biochemical analysis indicated that the total concentration of nucleotides (studied through TPAN) in haddock eggs did not differ between the two dietary groups. This phenomenon suggests that nucleotides were not stored and then 'loaded' into the eggs but instead utilised by broodfish in order to fulfil their nutritional requirements prior to spawning. Since nucleotides participate in all major metabolic pathways, a more complex mechanism of action involving other nutrients affected by nucleotides is suggested and further study is required.

During oogenesis, lipids are mobilised from the liver into the gonads in order to be incorporated into the oocyte during vitellogenesis. Studies in mammals have demonstrated that dietary nucleotides stimulate the hepatic synthesis of lipoproteins (e.g. Sánchez Pozo *et al.*, 1986; 1995; Morillas *et al.*, 1994) as a result of higher levels of apolipoproteins (Sánchez Pozo *et al.*, 1994). As explained in chapter 2, it could be suggested that dietary nucleotides also stimulated lipoprotein synthesis in fish, increasing the mobilisation of lipids from the liver towards the ovaries; this may help explain the significantly lower hepatosomatic index after spawning in haddock females from the nucleotide group. It is therefore hypothesised that higher nucleotide availability together with an increased mobilisation of lipids from the liver into the oocytes during oogenesis lead to an improvement of the reproductive performance in the broodstock fed the nucleotide-enriched diet. Spawning performance was affected by the nucleotide-enhanced

diet since relative fecundity in halibut and batch relative fecundity in haddock were significantly higher when broodstock were fed the nucleotide-enhanced diet.

Since broodstock reserves are finite, fecundity and egg size are two parameters that tend to be inversely correlated (Blaxter, 1969). However, in our study significant correlations between these two parameters were not found in halibut or haddock. Nevertheless, it is interesting that in the halibut group with the highest fecundity (the nucleotide group), the eggs were smaller when compared to those from the control diet. In haddock, the fact that several broodfish were reared in the same tank and spawned batches which could have originated from more than one female and were collected together made it more difficult to find a clear relationship between fecundity and egg size in this species.

6.3 Egg quality and physical characteristics

Dietary nucleotides also affected egg quality and their physical characteristics in both species. Improvements in halibut egg quality with the nucleotide-enriched diet were only reflected in improved hatching rates, while blastomere morphology and fertilisation rate (FR) were not significantly different between diets. The similarity of FR between control and nucleotide diets was in agreement with the results revealing that sperm motility, which decreased during the season in both groups, was also not affected by dietary treatment. The improvement in hatchability may have been influenced by the egg physical properties since some of them differed between diets. Thus, eggs from broodstock fed the nucleotide-supplemented diet had significantly higher density, dry weight and significantly lower water content.

As in halibut, the hatchability of haddock eggs was significantly improved by feeding broodstock with the nucleotide diet in the two spawning seasons tested. Fertilisation rate was also significantly improved with the nucleotide broodstock diet, although only during the first spawning season. Mean egg size was significantly larger in the nucleotide group in the two

consecutive spawning seasons and similarly the nucleotide eggs were significantly heavier, in terms of dry weight, than control eggs during the second spawning season.

The fact that halibut and haddock egg dry weights were significantly higher in the nucleotide group (the latter in the second spawning season only) may indicate that the transfer of material from the females into the eggs was possibly greater in the nucleotide-fed broodstock. This additional material transferred into the nucleotide eggs constitutes as an extra source of nutrients for the developing embryo and also yolk-sac larvae until exogenous feeding starts.

6.4 Larval morphometrics and growth

Halibut larvae from nucleotide eggs were significantly smaller, in terms of length and weight, when compared to control larvae at hatch. However, when egg size differences were taken into account, it was shown that larval size at hatch did not significantly differ between treatments indicating that nucleotide larvae were smaller due to the smaller size of the nucleotide eggs. However, despite their smaller size, the condition of the nucleotide larvae at hatch was never compromised and similar to that of control larvae. Furthermore, despite being smaller and lighter at hatch, nucleotide larvae caught up with control larvae in length and weight by the middle of the yolk-sac stage (126 $^{\circ}$ days), indicating that nucleotide larvae grew faster during this period. In fact, when larval length was corrected for egg size, the nucleotide larvae were significantly larger than those from the control group (i.e. grew faster than the control larvae). By the end of the trial (252 $^{\circ}$ days after hatch), all larval morphometric parameters were similar between diets. The nucleotide larvae remained larger than control larvae when egg size differences were taken into account, but within the same range than at 126 $^{\circ}$ days, indicating that nucleotide larvae grew faster only during the first half of the yolk-sac stage.

In haddock however, egg size differences were not initially reflected in the larval size at hatch and most morphometric parameters of the larvae were similar in both diets during the two

consecutive spawning seasons. An exception to this was a significantly larger yolk-sac volume in nucleotide larvae in the 2003 spawning season (also when corrected for egg volume). Larval trials were terminated five days after hatching in the 2002 spawning season and larval size by length was similar between control and nucleotide treatments at this stage. However, nucleotide larvae were significantly heavier which may indicate that they grew faster, as with halibut nucleotide larvae. During the 2003 spawning season, the trial terminated 10 days after hatching and nucleotide larvae were significantly larger, in terms of length and myotome height, and also had significantly higher growth rates when compared to the control group. The growth improvements in halibut and haddock larvae observed in this project are similar with previous fish studies that report nucleotide diets fed directly enhance growth, both in terms of size and weight, in seabream larvae (Bordá *et al.*, 2003).

6.5 Larval survival

As reported in chapter 4, halibut larval survival at the end of the yolk-sac stage (252 ° days post hatch) was significantly higher in the nucleotide group. Conversely, haddock survival rates were very similar at the end of the yolk-sac stage (5 DPH) between dietary treatments in 2002 and were almost significantly different ($p = 0.1$) at 10 DPH in 2003. Energy charge values of halibut larvae 252 ° days were similar between diets (around 0.5 – 0.55), which indicates that larvae were severely stressed by this time (Ivanovici, 1980). Haddock larvae presented high EC values (0.8) at the end of the yolk sac stage, indicative of larvae in normal condition. A possible explanation for the difference in EC ratio between both species at the end of the yolk-sac stage regarding the timing in the utilisation of the different nutrients was suggested in chapter five. Halibut larvae only use FAA and proteins during the first half of the yolk-sac stage (Finn *et al.*, 1995a) while haddock larvae utilised endogenous FAA but also phosphatidyl choline and triacyl-grycerol at hatch as cod (Finn *et al.*, 1995b). It is interesting to note that larvae with low EC values (halibut: 0.5-0.55) suffered high mortalities (>50%) while in larvae with high EC values (haddock: 0.8) the survival was very high (>80%). Halibut larvae should be provided

with live food from 200 ° days post hatching (Lein & Homelfjord, 1992; Finn *et al.* 1995a). However in the present study they were left without food until 252 ° days post hatching, therefore larvae from both dietary treatments were under nutritional stress conditions (confirmed by the low EC values). Although control and nucleotide larvae had similar EC values (C: 0.50 ± 0.08; NT: 0.55 ± 0.09) the survival of nucleotide larvae was significantly higher than in the control group. The growth and survival improvements reported in the nucleotide larvae are indicative of an enhancement in the larval quality for both species and this was also confirmed by the findings on gut development and first-feeding success in haddock larvae in the 2003 spawning season.

6.6 Gut development and first feeding success

Beneficial effects of nucleotide supplementation of broodstock diets were also observed with regard to larval gut development and feeding success. Hamlin *et al.* (2000) described the development of the digestive tract in haddock larvae and concluded that the development of the pyloric caeca, the last morphological change in intestinal development, occurs around 35 days post hatch. This is far beyond the duration of the larval trial in 2003, therefore the guts were not fully developed by 10 DPH. However, differences in larval gut development from broodstock fed the control or the nucleotide diets were observed. Guts of the nucleotide larvae were significantly more developed than those of the control larvae ten days after hatch. As discussed in chapter 4, these findings are consistent with the extensive literature reporting the beneficial effects of dietary nucleotides on gut development in different vertebrates, including fish (e.g. Burrells *et al.*, 2001b). Recently, Bordá *et al.* (2003) reported that dietary nucleotides improve intestinal development also in larvae (seabream). Gut development and first feeding success are two parameters which are directly linked. In fact, they were correlated in our study and nucleotide larvae also exhibited a significantly higher feeding success when compared to control diet. Based on this evidence it is suggested that the addition of extra nucleotides in broodstock feed led to an improved and accelerated gut development in the larvae so they started feeding on

rotifers earlier, grew better and it could also be inferred that survival was greater at 10 days post hatch. However, this effect was indirect since no enhancement of nucleotides in larvae occurred.

6.7 Adenine nucleotides and energy charge

The main goal of this part of the study was to study potential differences in the adenine nucleotide levels and energy charge between the control and nucleotide groups. Analysis of the adenine nucleotide content in egg and larval samples did not reveal any significant difference between diets in either species. Energy charge ratios in eggs and larvae were also similar in halibut and haddock samples, however the EC ratio of fertilised halibut eggs was significantly higher in the nucleotide diet. Energy charge in eggs decreased during the two consecutive haddock spawning seasons, although this was not observed in halibut eggs. Energy charge values from haddock embryo samples appeared to be correlated with the proportion of floating (viable) eggs in 2003, which is consistent with the results from Boulekbache *et al.* (1989) in which carp embryo mortalities increased with decreasing EC.

Additionally, spare egg and larval samples from the first haddock season were used to quantify their total nucleotide content (expressed as TPAN). This type of analysis was not carried out in halibut samples or haddock samples from 2003 because of insufficient spare samples, so results should be considered with caution. Total nucleotide levels were not higher in the eggs and larval samples from the nucleotide group. This finding may suggest that it was not nucleotides, *per se*, but other nutrients affected by nucleotides metabolically which improved egg and larval quality in the nucleotide group. Finding the active mechanism of dietary nucleotides on fish gonads was beyond the aims of this project. The fact that nucleotides participate in all major metabolic pathways suggests that these compounds may influence fish reproduction directly or indirectly at different levels.

Conclusions

Up until now most of the studies involving nucleotide diets and fish had focused on their effects on the immune system and/or growth performance (Table 1. 3). However, this project was innovative, since it was the first study to focus on the effects of nucleotide-enriched diets on fish reproduction. The findings presented in this thesis are consistent with those described by many scientists before but first reported by Watanabe (1985): that nutritional quality of the broodstock diet affects fish reproduction. Nucleotide broodstock diets consistently improved spawning performance, and egg and larval quality in Atlantic halibut and haddock, two species with different life histories. Feeding haddock broodstock with a nucleotide-enriched diet had no negative long-term effects on the broodfish condition and their reproductive performance. Dietary nucleotides are suggested to be responsible for the HSI values obtained in haddock at the end of the second spawning season: they were significantly lower and closer to values found in wild fish when compared to the control group. Further investigations into the potential effects of dietary nucleotides on lipid metabolism in fish should be carried out.

Future experiments should focus on exploring the use of higher levels of nucleotide supplementation in order to find the optimal inclusion levels in a long-term study. A further step could be to examine the optimal levels of each individual nucleotide in the diets of broodstock and rapidly growing juveniles in different species. Effects of nucleotides/nucleosides on oocyte maturation in marine teleosts would also be of much interest. It is now important to use all these findings as a benchmark for future studies on different fish species and to determine whether the same effects are obtained.

Appendices:

Appendix I: Sampling and planning during each spawning season.

A) 2001 halibut season:

Unfertilised eggs:

- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. From all the fish stripped. Spare samples were used to study egg **dry weights**.
- 3x egg counts in 2.5 ml of egg sample.

Milt:

- **Milt motility** assessment under binocular microscope (x3).

Fertilised eggs:

- **Fertilisation rate** and developmental stage determination of all batches (3x).
- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. From all the fish stripped.
- **Drop-outs** and number of **eggs/g** determination (3 counts) (all the batches).
- Microwell plates incubation (1 egg and 200 µl seawater in each well)=> **Blastomere morphology index** and **Hatching rates.**

At hatch:

- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. (5 larvae per vial).
- 3x 1ml-eppendorf for **dry weight. (5 larvae per vial).**
- Image grabbing (x3 magnification) of at least 5 larvae per batch for **morphometrics.**
- Set up **bowl** with ~200 larvae for **survival rate estimation.**

Half way of yolk-sac stage (21 days after hatching):

- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. (5 larvae per vial).
- Image grabbing (x3 magnification) of at least 5 larvae per batch for **morphometrics.**

End of yolk-sac stage (42 days after hatching):

- Yolk-sac **larval survival rates** from each bowl.
- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. (5 larvae per vial).
- 3x 1ml-eppendorf for **dry weight (5 larvae per vial).**
- Image grabbing (x3 magnification) of at least 5 larvae per batch for **morphometrics.**

B) 2002 haddock season:

Fertilised eggs straight after collection:

- **Fertilisation rate** and developmental stage determination of all batches (3x).
- Image grabbing of at least 5 eggs per batch for **morphometrics.** 1picture at x1.5 magnification and 2 at x3 magnification. (all batches)
- No. of **eggs/g** determination (1 count of 50 eggs and weigh them) (all the batches)
- 6x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. From 2 different tanks every day, one control and one nucleotide.
- 3X 1ml-eppendorf for **dry weight.** From 2 different tanks every day, one control and one nucleotide.
- **Drop-outs**
- Well plates incubation (50 µl sample/2ml seawater in each well)=> **Hatching rates. Prepare 2 plates per batch.**

At hatch:

- 6x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. (20 larvae per vial)

- 3x 1ml-eppendorf for **dry weight. (5 larvae per vial)**
- Image grabbing (x3 magnification) of at least 5 larvae per batch for **morphometrics**
- Set up **bowl** with ~200 larvae for **survival rate estimation**
- Set up **bowl** with ~100 larvae, 10ml algae for **first-feeding success experiments.**

End of yolk-sac stage (5 days after hatching):

- Yolk-sac **larval survival rates** from each bowl.
- 6x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. (20 larvae per vial)
- 3x 1ml-eppendorf for **dry weight (5 larvae per vial)**
- Image grabbing (x3 magnification) of at least 5 larvae per batch for **morphometrics**

C) 2003 haddock season:

Fertilised eggs straight after collection:

- **Fertilisation rate** and developmental stage determination of all batches (3x).
- Image grabbing of at least 5 eggs per batch for **morphometrics.** 1picture at x1.5 magnification and 2 at x3 magnification. (One control and one nucleotide batch every day)
- No. of **eggs/g** determination (1 count of 50 eggs and weigh them) (One control and one nucleotide batch every day)
- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. From 2 different tanks every day, one control and one nucleotide.
- 3x 1ml-eppendorf for **dry weight** with 10 eggs. From 2 different tanks every day, one control and one nucleotide.
- **Drop-outs**
- Well plates incubation (50 µl sample/2ml seawater in each well)=> **Hatching rates. Prepare 2 plates per batch.** (One control and one nucleotide batch every day).

At hatch:

- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. (20 larvae per vial)
- 3x 1ml-eppendorf for **dry weight. (5 larvae per vial)**
- Image grabbing (x3 magnification) of at least 5 larvae per batch for **morphometrics.** (One control and one nucleotide batch every day)
- Set up **vessel** with ~270 larvae, 50ml algae and rotifers for **survival rate estimation** and **first-feeding success experiments** (3x).

First Feeding Larvae (10 days after hatching)

- **Larval survival rates** from each vessel.
- **First-feeding success** in larvae. Check for gut contents.
- 3x 1ml-vial for **nucleotide analysis** to keep in liquid nitrogen. (20 larvae per vial)
- 3x 1ml-eppendorf for **dry weight (5 larvae per vial)**
- Image grabbing (x3 magnification) of at least 5 larvae per batch for **morphometrics**

Appendix II: Record of mortalities in haddock broodstock nutrition trial

The following tables (I to VI) describe mortality in each of the six trial tanks since the end of the spawning season in 2002. In the tables, the 'Ardtoe refs' correspond to fish health sheets and Fish Vet Group laboratory reports. The relevant comments below each table summarise these reports.

Table I: Mortalities in Tank F1 (Control diet)

Date	Sex	Number	Ardtoe ref.	Cumulative mortality
25.07.02	1 ♂	1	226	1
03.10.02	1 ♀	1	319	2
20.10.02	1 ♂	1	323	3
15.12.02	2 ♂	2	376-377	5
16.12.02	4 ♂	4	379-382*	9
17.12.02	1 ♀	1	390A	10
18.12.02	4 ♂ & 2 ♀	6	391-396*	16
20.12.02	1 ♂	1	403	17
22.04.03	1 ♀	1	?	18

* The fish health sheet for ref. 379 describes extensive haemorrhaging on all fins. Refs. 391 to 396 catalogue six mortalities. Reasons for death are unclear.

Table Ii: Mortalities in Tank F3 (Control diet)

Date	Sex	Number	Ardtoe ref.	Cumulative mortality
11.07.02	?	1	207	1
11.09.02	♂	1	258	2
30.09.02	♂	1	304	3
01.10.02	1 ♀ & 1 ♂	2	315-316	5
02.10.02	♀	1	318	6
14.10.02	?	1	320	7
02.11.02	♂	1	332	8
03.12.02	?	1	351	9
07.12.02	♀	1	372	10
22.01.03	?	1	412	11
10.04.03	?	1	?	12

Table III: Mortalities in Tank R2 (Control diet)

Date	Sex	Number	Ardtoe ref.	Cumulative mortality
19.06.02	1 ♀	1	199	1
16.09.02	1 ♀	1	269	2
05.12.02	1 ♀	1	352	3
11.01.03	1 ?	1	?	4

Table IV: Mortalities in Tank F2 (Nucleotide diet)

Date	Sex	Number	Ardtoe ref.	Cumulative mortality
15.06.02	1 ♀	1	195	1
16.06.02	1 ?	1	198	2
06.08.02	1 ♂	1	231	3
22.08.02	1 ♂	1	245	4
09.09.02	2 ♂	2	255-256*	6
17.09.02	1 ?	1	271	7
02.10.02	1 ♂	1	317	8
01.11.02	1 ♂	1	331	9
18.11.02	1 ♂	1	338	10
08.12.02	1 ♀	1	373	11
15.12.02	1 ♀	1	375	12
16.12.02	1 ♂	1	378	13
10.02.03	1 ?	1	?	14

*The health report (case ref. 566-02, sample 255) describes degenerative changes in the kidney, which may indicate absorbed toxin and/or hypoxia. No episode of hypoxia was recorded in the oxygen monitoring system.

Table V: Mortalities in Tank R1 (Nucleotide diet)

Date	Sex	Number	Ardtoe ref.	Cumulative mortality
12.09.02	♀	1	260*	1
13.09.02	3 ♂	3	261-263*	4
14.09.02	2 ♂ & 2 ♀	4	264-267	8
15.09.02	1 ♂	1	268	9
17.09.02	2 ♀	2	273-274	11
18.09.02	5 ♂, 1 ♀ & 2 ?	8	275-282*	19
19.09.02	5 ?	5	284-288	24
20.09.02	1 ♂	1	290	25
16.10.02	1 ?	1	321	26
17.01.03	1 ?	1	410	27
31.03.03	1 ♀	1	?	28

* The health report (case ref. 566-02, sample 260 & 261) describes degenerative changes in the kidney, which may indicate absorbed toxin and/or hypoxia. No episode of hypoxia was recorded in the oxygen monitoring system. *Vibrio* was suspected as the cause of mortality for refs 275-282. The fish were placed on medicated feed, and mortality decreased after the 19th September.

Table VI: Mortalities in Tank R3 (Nucleotide diet)

Date	Sex	Number	Ardtoe ref.	Cumulative mortality
01.11.02	♀	1	330	1
02.11.02	♂	1	333	2
03.11.02	2 ♂	2	334-335	4
06.12.02	9 ♂& 5 ♀	14	353-367*	18
07.12.02	2 ♂	2	370-371	20

* The health report (case ref. 773-02, Ardtoe ref. 353-367) report on *Costia*-like organisms (CLO) found on moribund fish. The finding of CLO's and associated pathology was attributed as the primary cause of the event mortality.

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